# STRUCTURES OF COMPLEX PLANT POLYSACCHARIDES

## Exudates from Hakea sericea and Hakea gibbosa

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**by**

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**ABSTRACT**

The polysaccharide exudates from two species of Hakea (fam. Proteaceae), H. sericea (from Grahamstown) and H. gibbosa (from Constantiaberg), have been investigated. In this study molecular structural differences which may arise from the species of origin were sought. The possibility that a polysaccharide component of the glucuronomannan type might be present was of interest, as this structure is rare.

These polysaccharides were analysed by a combination of the classical, standard methods, and the newer methods including those which utilise sophisticated spectroscopy. The rationale for choice of methods was that, as in the study of H. acicularis which is used for comparison in this study, the core structure was probably composed of glucuronic acid, mannose and galactose residues surrounded by xylose, arabinose and galactose units. The classical methods used included partial acid hydrolysis followed by column chromatography of the products, Smith degradation and methylation analyses. Smaller quantities of samples, lability of certain sugars to acid, the presence of glucuronic acid and the stability of the bonds linking this to other sugars necessitated the use of group-specific or selective degradative methods. These included the degradation of glucuronic acid residues by bases such as DBU and potassium-dimsyl, and partial hydrolysis with acid, which yielded products characterised by g.l.c.-m.s. and

n.m.r. ($^{1}H$ and $^{13}C$) utilising 1-D and 2-D methods. F.a.b.-m.s. (positive and negative modes) permitted the analysis of oligomers isolated by partial hydrolysis of polysaccharides.

These two Hakea polysaccharide gums have been shown to have close structural similarities. Each contains a core structure of glucuronic acid and mannose surrounded by an arabinoxylogalactan envelope. Their component sugars are mainly galactose, with a smaller proportion of arabinose, and mannose and xylose (molar ratio 1:1) as minor components. Glucuronic acid and mannose were found in equal proportion in H. sericea gum and in molar ratio 2:1 in that of H. gibbosa. Smith degradations and partial hydrolyses indicated that mannose and glucuronic acid residues become concentrated in products isolated from the innermost core, and occurring in a molar ratio of 1:1 in the core itself.

The cores of the H. sericea and H. gibbosa gum polysaccharides, composed of galactose, mannose and glucuronic acid, were shown to be very similar. However, the following differences in structure were noted :

i. Some xylose was present in H. sericea gum, probably linked to O-3 of glucuronic acid;
ii. The proportion of glucuronic acid appeared to be double that of mannose in the native polysaccharide of H. gibbosa, whereas these residues were equimolar in H. sericea gum;

iii. The glucuronic acid residues in the native polysaccharide of *H. gibbosa* gum were mainly 3,4-linked, whereas an equimolar proportion of 4-linked glucuronic acid was found in *H. sericea* gum;

iv. *H. gibbosa* gum contained 50 % more arabinofuranosyl units present as end group.

An "average" structure for the polysaccharide gum exudate from the two *Hakea* spp is proposed.

A publication which includes part of the work reported in this thesis is "Mannoglucuronomannoglycans from plant sources" by A.M. Stephen, P.F.K. Eagles, W.T. Mabusela, D.C. Vogt and A.M. Lawson, *Food Hydrocolloids*, 5 (1991) 159-161.

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To Maureen.

## ABBREVIATIONS

| | |
|---|---|
| ca. | approximately |
| Ara | arabinose |
| c | concentration |
| cm | centimetre |
| d | day |
| D | Deuterium |
| $d_2$ | deuterated |
| DBU | 1,5-diazabicyclo [5.4.0] undec-5-ene |
| DSS | sodium 4,4-dimethyl-4-silapentane-1-sulfonate |
| dimsyl | methylsulphinylmethanide, $CH_3SOCH_2^-$ |
| e.i.m.s. | electron-ionisation mass spectrometry |
| Ery | erythritol |
| EryA | erythronic acid |
| eV | electron volt |
| f | furanosyl |
| f.a.b. | fast atom bombardment |
| f.a.b.m.s. | fast atom bombardment mass spectrometry |
| g | gram |
| Gal | galactose |
| g.l.c. | gas liquid chromatogaphy |
| g.l.c.-m.s. | gas liquid chromatogaphy mass spectrometry |
| Glc | glucose |
| GlcA | glucuronic acid |
| h | hour(s) |
| h.p.l.c. | high performance liquid chromatography |

| | |
|---|---|
| Hz | Hertz |
| i.d. | internal diameter |
| i.r. | infrared |
| L | litre(s) |
| M | molar |
| m | metre |
| Man | mannose |
| m/z | ratio of mass to electron charge |
| min | minute |
| mL | millilitre [ml is used in the figures of steric-exclusion chromatograms] |
| mol | mole |
| mmol $g^{-1}$ | millimole per gram |
| mol.wt. | molecular weight |
| m.s. | mass spectrometry |
| $M_w$ | weight-average molecular weight |
| n.d. | not determined |
| nm | nanometre |
| n.m.r. | nuclear magnetic resonance |
| p | pyranosyl |
| p.c. | paper chromatography |
| p.m.a.a.(s) | partially methylated alditol acetate(s) |
| p.p.m. | parts per million |
| SD | Smith degradation |
| s | singlet |
| s.e.c. | steric exclusion chromatography |
| T | terminal |

| | |
|---|---|
| TFA | trifluoroacetic acid |
| THF | tetrahydrofuran |
| TMS | trimethylsilyl, $(CH_3)_3Si$ |
| tr | trace |
| Vo | Void volume |
| V | total volume |
| v/v | volume per volume |
| vol(s) | volume(s) |
| w/w | weight per weight |
| Xyl | xylose |

Abbreviations omitted from this list are defined in the text.

**NOTES TO READERS**

1. The term "autohydrolysis" refers to partial acid hydrolysis, at pH 1.7.

2. Naming of methyl ethers: in most cases the methyl (Me) has been omitted and only the numbers which refer to the positions where they occur have been used eg. 2,3,4-Gal is 2,3,4-tri-O-methyl-D-galactose, etc.

3. Condensed forms of naming oligosaccharides have been used in the text and tables, thus Gal-6 Gal is β-D-Galp-(1-6)-D-Gal) or 6-O-β-D-galactopyranosyl-D-galactose, etc.

4. The preparation and characterization of 2-O-β-D-glucuronopyranosyl-D-mannose in the investigation of the polysaccharides from Hakea sericea and H. gibbosa gums precedes in the text the partial hydrolysis of each of these polysaccharides, because the presence of the GlcA-Man had to be established first in order, to justify the search for the oligomers of this aldobiouronic acid (by partial hydrolysis, which followed this step).

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# 1. INTRODUCTION

## 1.1. Occurrence, Properties and Applications of Gum Polysaccharides.

Polysaccharide gum exudates are produced by several hundreds of plant species, a few from the gymnosperms and the rest from more than 25 of the 92 orders of angiosperms[1]. They are produced by trees either spontaneously, due to bacterial attack or after mechanical trauma[2]. Ease of isolation of polysaccharides from these exudates varies and some of the well-documented questions or problems are (i) variability in sample composition, (ii) homogeneity[3] and (iii) the special difficulty of analysis which results from the sensitivity to acid of some units in the polysaccharide and also the resistance to hydrolysis of aldobiouronic acid and mutually linked uronic acid units.

The analytical procedures used to determine monosaccharide composition, linkages between these sugars and eventually the structure of the gum polysaccharide include the so-called classical methods[4,5] and also the newer approach of targeting special areas or groups in the polysaccharide for treatment, e.g. base degradation of uronic acid units as esters.

Both methods as far as possible utilise sophisticated techniques such as n.m.r. of the 1-D and 2-D types and f.a.b.-m.s. to elucidate the structures of products.

The numerous properties of these polysaccharides have resulted in their many diverse and special applications in industries such as food and pharmaceutical manufacture, and mining. Applications are mainly the result of molecular characteristics such as size distribution, shape, degree of aggregation, changes in conformation with temperature, ionic strength of the medium and pH, and distribution of charged sites, hydrophobic regions and points of attachment to protein, as well as the degree of regularity of the macromolecules[1,6,7]; rheological properties are controlled by such molecular characteristics as these.

Important general properties that govern the uses of polysaccharides include :

(i) The emulsifying action, which is very important in the preparation of foods such as instant ready-prepared soups, icing, dressings and processed meats and in the manufacture of pharmaceutical dosage forms including suspensions and water-oil emulsions. Polysaccharides often used as emulsifiers are gums arabic and tragacanth, the latter being active at concentrations as low as 0.25 %.

(ii) The stabilising or thickening actions which increase the viscosity of the aqueous phase and so decrease the migration of the dispersed globules or particles, leading to the inhibition of coalescence, flocculation and sedimentation.

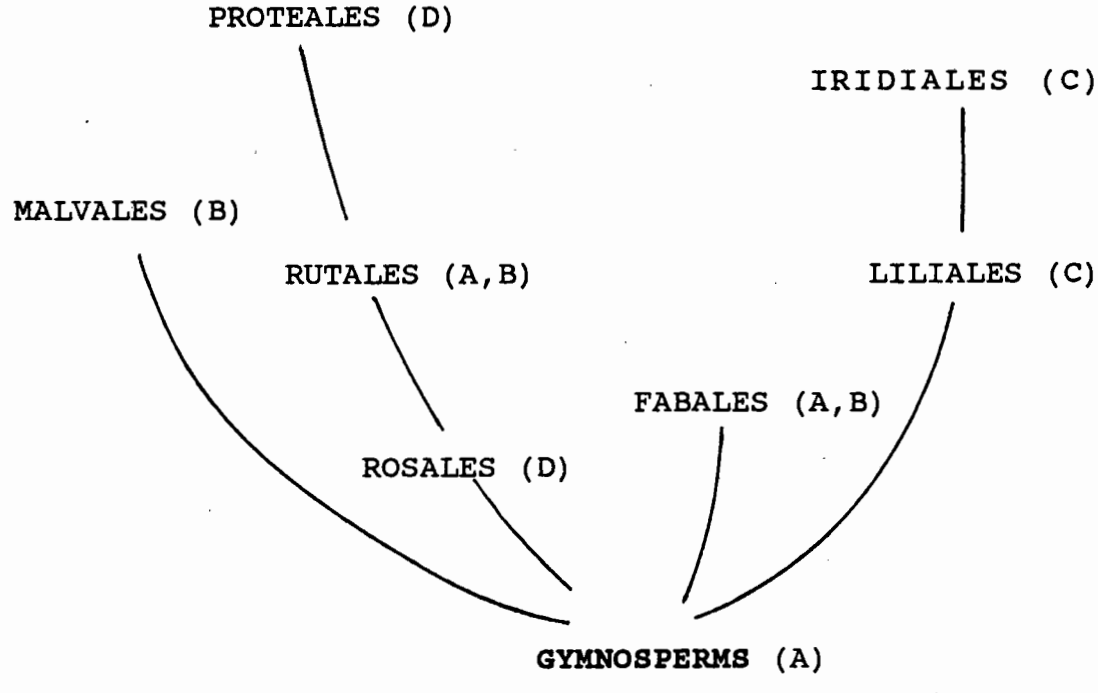
Generally in polysaccharide gums of high molecular weight and containing uronic acid there exist an area of multiplicity of hydroxyl groups, an area of relative hydrophobicity in the periphery, and a core. Bound protein and also association of protein with polysaccharides are increasingly becoming important considerations both in structural studies and in their application in food and pharmaceutical manufacturing (eg. in formulations of therapeutic parenteral nutrition fluids used for special hospitalised patients).

## 1.2. Structures and Taxonomy of gum-producing plants

In general polysaccharides are heteroglycans with the innermost main chains of the cores consisting of substituted D-glucans, D-mannans, D-galactans, D-galacturonans or D-xylans. Even though the recognition of these different structures has led to the classification of polysaccharides as types A,B,C[1], and D[8,9] it has become apparent that there is no clear discontinuity in polysaccharide structure between, for example, mannans and glucomannoglycans, which have been

shown to merge; both carry pendant galactosyl groups in proportions that vary greatly[1]. However, gums of various structural types are concentrated within certain orders and families, and within a genus they contain the same constituent sugar units and their modes of linkage are similar, but they are specific for given species. The structures of gums are affected only to a small degree by the conditions and geographical area of growth and also the part of the plant from which gum is collected. Some of these minor changes could be due to variations in the conditions under which biosynthesis occurred.

The major orders from which gum exudates of the different structural types (given in parentheses) have been isolated are presented[6] :



The different structural types of polysaccharides are now discussed briefly.

**Type A** polysaccharides, such as larch gum (Larix spp) and those from Acacia spp[7] have complex structures composed of a 3,6-linked D-galactopyranose core structure to which are attached, directly, single units or chains of L-arabinofuranosyl, or, exteriorly, chains of L-Rhap residues attached to O-4 of GlcA which in turn are linked to O-4 or O-6 of D-Gal.

**Type B** polysaccharides have a core structure composed of (1-4)-linked D-GalA with varying amounts of (1-2)-linked L-Rhap ranging from a low amount in tragacanth gum (Astragalus spp)[10,11] to a situation where it alternates with Galp in the major part of the structure in gum karaya from Sterculia urens[12-15]. Also present are short chains composed of D-Galp, L-Araf, D-Xylp, L-Fucp and D-GlcpA.

**Type C** polysaccharides, found in only a few plant species, are composed of a (1-4)-linked D-xylan core structure with numerous single unit substituents such as Araf, D-GlcpA and D-Galp attached to O-2 and O-3 positions in the core[16].

**Type D** polysaccharides contain the characteristic glucurono-mannoglycan core structure composed of alternating residues of (1-2)-linked mannose and (1-4)-linked GlcpA[1,16-18].

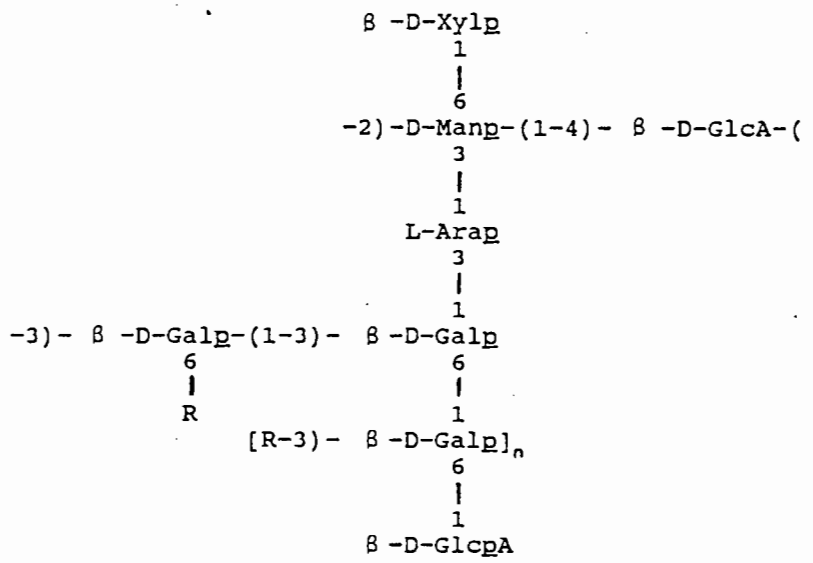
Substituents of this structure are heavily branched side-chains, structurally resembling the type A polysaccharides, which are attached to O-3 of most Man and sometimes O-3 of GlcpA residues. Examples of type D polysaccharides are presented.

(i) Gums from Anogeissus spp (Combretaceae)

The type D core structure was first detected in the polysaccharides from A.latifolia (gum ghatti)[19] and A. leiocarpus (leiocarpan gum)[20] and was structurally elucidated by Aspinall and his group.

In gum ghatti the glucuronomannoglycan core, shown to be present by acetolysis[21], has attached to it side-chains of mostly (1-6)-linked D-Gal units, some terminated by GlcA, joined to the Man units at O-3 through (1-3)-linked L-Arap residues. This linkage was verified by the isolation of the 3-O-L-arabinopyranosyl-D-mannose component after partial hydrolysis of the Smith-degraded product. Araf occurs as terminal groups or short sequences, with intersugar linkages between them being (1-2), (1-3) and (1-5), attached at O-3 of 6-linked Gal in the side-chains and some at O-6 of the Man units (double branch points)[19]. D-xylose occurs as a minor component in gum ghatti.

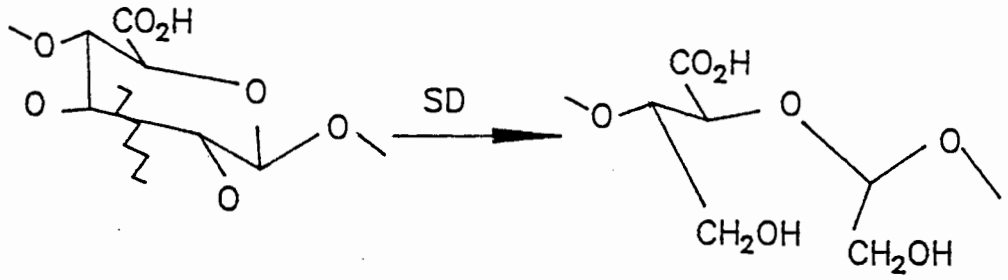
Leiocarpan A, the main component of the gum from A.leiocarpus was isolated as the less-soluble fraction by differential cetyltrimethylammonium bromide fractionation. Its glucurono-mannoglycan core was established after characterisation of the products obtained from acetolysis of the carboxyl-reduced polysaccharide. However leiocarpan is different from gum ghatti, mainly because of the presence of appreciable side chains of (1-3)-linked Galp. Base degradation studies, utilising DBU as catalyst, on the methylated autohydrolysed (partially hydrolysed) leiocarpan A, followed by borohydride reduction, acetylation and g.l.c-m.s. of the remethylated products, indicated, as in gum ghatti, the presence of single β-D-xylopyranosyl (end groups) at O-6 of Man[20,23,24]. The β-L-arabinofuranose unit at O-3 of Man was shown by the lead acetate decarboxylation-acetoxylation procedure[25,26]. A representation of some of the structural elements follows[1] :




The value of n is much lower than in gum ghatti. R could represent T-L-Araf or Araf units linked (1-3).

(ii) Gums from Prunus spp (Rosaceae)

Gums from P.cerasus (cherry gum)[27], P.armeniaca (apricot gum)[28] and P.spinosa (blackthorn)[29] all have properties which probably classify them as type D polysaccharides. The absolute proof for the alternating GlcA and Man residues in cherry gum[27] is not yet available. It was shown that there are branches of 6- and 3,6-linked Gal attached to O-3 of Man and of some of the GlcA residues. Smith degradation of partially hydrolysed apricot gum required prolonged treatment with M TFA at room temperature to cleave the acetals formed by substituted erythronic acid units, produced on oxidation and reduction of the 4-linked GlcA units, and the glycolaldehyde also produced; these resistant acetal linkages (1) were interspersed between the periodate-immune blocks of sugars. G.l.c. after total acid hydrolysis, reduction and acetylation indicated the presence of erythritol equivalent in amount to the degraded GlcA[28].

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(iii) The gum of Encephalartos longifolius (Zamiaceae)

This polysaccharide gum was shown to contain sequences of GlcA-2Man in the core structure with substitution by 3-linked Gal chains on to the O-3 of Man, either directly or indirectly through 3-linked Arap[30-32]. O-4 of mannose is substituted by sugar residues, some of which are resistant to acid hydrolysis and some being resistant to periodate oxidation. The substituent on O-3 of GlcA is probably the acid-labile Araf. Another substituent which could have significance as a chemotaxonomic marker is 3-O-methyl-L-rhamnose, which appears to be located close to the core of the polysaccharide. The core structure was established when $(GlcA\text{-}2Man)_n$, with n ranging from 1 to 4, was liberated on partial hydrolysis. F.a.b.-m.s. of methylated, degraded gum

fractions in the mol.wt. range 4400-1400 confirmed the presence of sequences with n $\leq$ 5. GlcA also occurs in the periphery attached to galactose at its O-6 position and interior to rhamnose as found in Acacia and other plant gums.

The core structure of E.friderici-guilielmi gum was also shown to be of type D[32].

(iv) Gum from Chorisia speciosa[17]

Methylation analysis and selective alkaline degradation, combined with the characterization of oligosaccharides liberated by partial hydrolysis, allowed a tentative average structure to be assigned. The glucuronomannoglycan core was again substituted at the O-3 of mannose by galactan side chains of 3-linked galactose and 3,6-linked Galp branch-points carrying mainly 6-linked galactose units with some in-chain 4-linked GlcA; side-chains terminated with galactose, arabinose, glucuronic acid or rhamnose units. Mannose occurred only in the core structure and was not further substituted.

(v) Gum of Grevillea robusta. (Proteaceae, silk oak) [33,34]

A sequence of experiments including partial hydrolysis, Smith degradation with subsequent prolonged exposure to M TFA for complete degradation of the glycolaldehyde-linked erythronic

acid bridge(s) between the periodate-resistant blocks of sugars, and DBU-catalysed degradation of the GlcA residues in the methylated product allowed postulation of a sequence of nine contiguous -4)-GlcA-(1-2)-Manp-(- units in the core of this polysaccharide. However, evidence exists for periodic interruption of the glucuronomannoglycan sequence by adjacent Manp residues. Further experimentation is required using selective cleavage of glucopyranosiduronic acid linkages and partial acid hydrolysis. The O-3 of mannose is again substituted by 3-, 4-, or 6-linked Gal to which arabinose and xylose are attached.

(vi) Gum of Brabeium stellatifolium (Proteaceae, wild almond [35,36]

This exudate gum is composed of units similar to those found in the exudate of G. robusta. The main structural features of the gum polysaccharide were found to be a basal structure of D-GlcA and Man with the latter (1-4)-linked to the GlcA residues. Smith-degradation studies revealed a periodate-resistant core of average size 20 hexose units. D-galactose side-chains were attached to the glucuronomannoglycan core and L-arabinose residues, mostly as terminal and some as chain units, were also present. D-xylose sometimes replaced arabinose as end-groups while the location of the L-rhamnose, present as a minor constituent, was not certain.

(vii) Mucilage from Ornithogalum thyrsoides (Hyacinthaceae)[37]

The cetavlon-precipitable acidic polysaccharide, isolated from the water-soluble mucilage of the leaves of the plant, released on partial hydrolysis only terminal Araf from O-3 of D-GlcA to yield a skeleton consisting of equal parts of 4-linked GlcA and 2-linked Man. This structure on further treatment with acid furnished aldobiouronic acid GlcA-2Man and oligomers thereof [$(GlcA\text{-}2Man)_n$ with n=2-5]. F.a.b.-m.s. of a per-O-methylated fraction having n>5 revealed the presence of oligomers having n=6, 7, 8 and 9, while $^{1}H$ and $^{13}C$ n.m.r. of the arabinose-free polysaccharide and the n=3 oligomer confirmed the type D core structure.

(viii) Mucilage from Actinidia deliciosa (Kiwi fruit)[18,38,39]

The polysaccharide, obtained from the mucilage of the stem pith, was shown on methylation analysis of the native, the carboxyl-reduced and the partially acid-hydrolysed polymer to have the glucuronomannoglycan core structure. Most of the D-mannosyl and approximately half of the D-GlcA residues were substituted at the O-3 positions with neutral oligosaccharides, composed of (1-3)-linked Gal residues partially substituted through positions 2 and 6 with Araf, Arap, Fucp and Galp. F.a.b.-m.s., e.i.m.s. and g.l.c-m.s. of the methylated alditol acetates of the hydrolysed methylated oligosaccharide, isolated after the polysaccharide had been

subjected to graded acid hydrolysis, confirmed the presence of the type D structure.

(ix) Polysaccharide from Nicotinia tabacum cells [40,41]

The extracellular polysaccharide from suspension-cultured cells of tobacco, after partial acid hydrolysis and n.m.r. analyses of the products of hydrolysis, once again showed the presence of a core composed of a regularly repeating structure of alternating Man and GlcA residues, each substituted with L-Araf at their O-3 positions.

(x) Polysaccharide from Lessonia nigrescens[9]

Partial hydrolysis of the polysaccharide from this brown seaweed followed by s.e.c. gave 4 fractions, viz. monosaccharides (not including Man), neutral (composed solely of Gal) and acidic oligosaccharides (these were inseparable), a soluble polymer (20 % of the starting material) and a small insoluble residue. The glucuronomannoglycan structure was found only in the soluble polymer which on methylation and n.m.r. ($^{13}C$ and $^{1}H$) was shown to be composed of a linear chain of ca. 44 alternating units of GlcA and Man.

(xi) Mucins from Drosera binata and D. capensis[9,42]

These are carnivorous plants the leaves of which contain polysaccharide mucins of the type D core structure substituted at the O-3 position of GlcA with Araf and Xylp and at the same position of Man with Galp.

Structural similarities to the gums presented in this discussion have been noted in polysaccharides from other plant sources eg. Virgilia oroboides (Papilionaceae)[43], Albizia zygia (Mimosaceae)[44] and Hakea acicularis (Proteaceae)[45].

## 1.3. The *Hakea* Genus : Classification

The family *Proteaceae* is distributed widely in Africa, Madagascar, Australasia, the Western Pacific, and Central and South America. The genus *Hakea*, a name given to a group of approximately 100 types of evergreen trees in honour of the baron Christian Ludwig von Hake (1745-1818)[46], is classified under the tribe *Grevilleae* [also including the genera *Finschia* and *Grevillea* (235 spp)], which is in the sub-family *Grevilleoideae* (40 genera)[47].

Polysaccharide chemistry was stimulated when the results of chemical analysis of the secondary products such as water-soluble gum exudates were used in systematic taxonomy. Data for *Hakea* and *Grevillea* species are still limited[34-36,48].

The only published investigation of a *Hakea* gum was a preliminary study of that of *H. acicularis*[45]. This species, referred to also as *H. tenuifolia*, has now been renamed *H. sericea* and a detailed study of its gum exudate, together with that of *H. gibbosa*, is presented in this thesis.

## 1.4 **Methodology of Polysaccharide Structural Studies**

Polysaccharides are analysed by a combination of the classical standard methods[1,5,6,49-53], the group-specific methods[21,54-56] and the newer methods including those which utilise highly sophisticated spectroscopic instrumentation[57-60]. The determination of monosaccharide composition, linkages between these sugars, anomeric configuration and eventually the structure of the polysaccharide has allowed classification of gum polysaccharides and application in chemotaxonomy [61,62].

The classical methods, viz. partial acid hydrolysis, column chromatography, Smith degradation[63,64,65], and methylation analyses[40,66-68] were used in the structural elucidation of many gum exudates. The H. acicularis gum exudate[45] was studied and the results are used for comparison in the present study. Smaller quantities of samples, lability of certain sugars to acid hydrolysis, the presence of uronic acid and the stability of the bonds linking it to other sugars have necessitated the use of group-specific or selective degradative chemical methods. These include the degradation of uronic acid residues by bases such as DBU[20,69] and potassium-dimsyl[70], and partial hydrolysis with acid, which yield products characterisable by g.l.c.-m.s. and n.m.r. ($^{1}H$ and $^{13}C$) utilising 1-D and 2-D methods[71-79].

More sophisticated analyses of the products, especially oligomers isolated by partial acid hydrolysis of polysaccharides, include methods used to some extent in the present study such as f.a.b.-m.s. (positive and negative modes)[79].

Recently there has been an increase in the use of HPLC in preference to the older column chromatography as a preparative tool for the quick, less laborious, economical and accurate analysis of oligomers, plain and derivatised (eg. methylated), but the obvious limitations here, as for many other more sophisticated applications of experimental procedures, include the large capital expense and running costs.

In the present study on the high molecular weight polysaccharides exuded from the two Hakea trees, the methods used included total and partial acid hydrolyses, methylation analyses, Smith degradation, the base-catalysed degradation of uronic acid residues, and column chromatography with analyses of the eluted products by various instrumental and spectroscopic methods. The rationale for the choice of these methods was mainly the probability[45] of a core structure composed of uronic acid, mannose and galactose residues surrounded by a larger envelope of xylose, arabinose and galactose units. The possibility of heteropolymolecularity of the polysaccharides from H. sericea gum exudate, based on the production of fractions differing in solubility in water, was also investigated by these approaches.

## 2. GENERAL EXPERIMENTAL CONDITIONS

### 2.1 PAPER AND THIN-LAYER CHROMATOGRAPHY

Paper chromatography (p.c.) was carried out on Whatman No.1 paper using the following solvent systems (all v/v):

A. 1-butanol-ethanol-water (4:1:5, upper phase),
B. ethyl acetate-pyridine-water (8:2:1),
C. 1-butanol-acetic acid-water (2:1:1) and
D. ethyl acetate-acetic acid-formic acid-water (18:3:1:4)

Preparative p.c. was performed on sheets of Whatman 3 MM paper, pre-eluted with the solvent system to be used, then washed with deionised water and finally air-dried. The sample was dissolved in water and applied in a thin band near the top of the sheet. Small aliquots of the sample solution were also applied to thin, separate strips which were developed at the same time as the main sheet, but removed successively at intervals to monitor the extent of migration of the components. After elution had been completed the positions of the components were determined by cutting and spraying test strips from either side and in the middle of the sheet. The bands were cut from the sheets and the components were eluted from the paper overnight with a

deionised water-methanol mixture (1:1 v/v). The solution was then filtered through a sintered glass funnel, concentrated and freeze-dried.

Thin-layer chromatography was carried out on Merck aluminium sheets pre-coated with silica gel 60 F, thickness 0.2 mm, using solvent C or E, a mixture of chloroform-methanol-water (20:20:7).

Reagents and techniques used for detecting components separated by p.c. and t.l.c. were as follows:

1. Spraying with a solution (ca. 3 % w/v) of p-anisidine hydrochloride in 1-butanol (water-saturated), followed by heating at 110° C for 5 to 10 minutes. This method was used routinely to detect sugars.

2. Dipping the paper successively through

   a. 0.6 % (w/v) solution of $AgNO_3$ in acetone followed by drying; and then
   b. 2 % (w/v) solution of NaOH in ethanol; the intensity of the background stain was reduced by washing finally with 30 % aqueous ammonia. This method allowed detection of sugars and polyols.

3. Carbohydrate was detected on t.l.c. (solvent E) by spraying with p-anisaldehyde-sulphuric acid-ethanol (1:1:18 v/v) followed by heating at 110° for 5-10 minutes.

$R_{Gal}$ values refer to the rate of movement relative to galactose.

## 2.2 GAS-LIQUID CHROMATOGRAPHY (g.l.c.)

G.l.c. analysis of alditol acetates or partially methylated alditol acetates (p.m.a.a.s) was performed on a Carlo-Erba 4200 gas chromatograph, coupled to a Columbia Supergrator-3A integrator for quantitative analysis, using the following columns:

Column A: glass column (2 m x 3 mm i.d.) packed with 3 % OV-225 on Chromosorb W-HP, 80-100 mesh, or Supelcoport, 100-120 mesh.

Column B: fused silica capillary column DB-225 (30 m x 0.32 mm i.d.), film thickness 0.25 microns (J & W Scientific, Inc.).

The carrier gas was helium. Mixtures of alditol acetates[80] were analysed on column A, isothermally at 170°C, and on column B isothermally at 215°C. Mixtures of p.m.a.a.s were analysed on columns A and B, isothermally at 215°C. Column B was used with a splitter injection system, 1:40 split ratio.

A flame ionisation detector at 300°C was employed. For g.l.c.-m.s. measurements an identical chromatograph was coupled through a jet separator to a V.G. Micromass 16F spectrometer, operated at 70 eV or at 20 eV. Plots of total ion-, single ion-, and selected ion-current were made against time. Columns A and B were used. Components in the mixtures analysed were identified by comparison of retention times with standards run under identical conditions, by co-injection of standards in some cases, and by g.l.c.-m.s. Quantitative analysis of mixtures of p.m.a.a.s was achieved by using the molar response factors of Sweet and Albersheim[81] or the molar response factors determined, immediately before and after the analysis, by injection of appropriate standards, and empirically determined molar response factors were used for mixtures of alditol acetates. Retention times $T_m$ are relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol.

## 2.3 FAST ATOM BOMBARDMENT-MASS SPECTROMETRY

The samples, in a matrix of thioglycerol, were analysed in a VG Analytical ZAB2-E mass spectrometer operated at 8keV accelerating voltage using a Cs ion gun (25keV, 0.5 A emission).

## 2.4 STERIC-EXCLUSION CHROMATOGRAPHY (s.e.c.)

Weight-average molecular weights ($\bar{M}_w$) were estimated by s.e.c.[82] using the following columns (all with M NaCl as eluent):

Column 1: Sepharose 4B (60 x 0.9 cm); for molecular weight range $5 \times 10^4$ to $> 10^6$ Daltons,

Column 2: Bio-Gel P-10 (52 x 1.5 cm), molecular weight range 300 to $> 10^4$ Daltons,

Column 3: Bio-Gel P-2 (55 x 2.5 cm); molecular weight range up to 1800 Daltons.

These columns were calibrated using Pharmacia characterised dextrans ($\bar{M}_w$ $2 \times 10^6$, $5 \times 10^5$, $1.5 \times 10^5$, $5.2 \times 10^4$, and $1.16 \times 10^3$), and standard oligosaccharides.

The presence of carbohydrate in the eluted fractions was monitored by the phenol-sulphuric acid method[83]. Preparative s.e.c. was performed on various columns which will be described in the text.

## 2.5 SAMPLE HANDLING AND SPECTROSCOPIC MEASUREMENTS

2.5.1 Solutions were concentrated at 40°C and 20 mm Hg on a rotary evaporator.

2.5.2 Optical rotations were measured from aqueous solutions for underivatized samples, and from chloroform solutions for methylated products, at 20°C (c 1 %) on a Perkin-Elmer Model 141 polarimeter.

2.5.3 Dialysis was carried out versus distilled water using Spectra/Por membrane tubing (Spectrum Medical Industries, Inc.) with molecular weight cut-off (i) 8000, (ii) 3500.

2.5.4 Infrared spectra of methylated derivatives in chloroform (c ~1 %) were recorded on a Perkin-Elmer Model 237 spectrophotometer.

2.5.5 90 MHz $^{1}H$ and 22.6 and 50.3 MHz $^{13}C$ n.m.r. spectra were obtained on a Bruker WH-90 spectrometer and 200 MHz $^{1}H$ spectra on a Varian VXR-200 spectrometer.

Samples were prepared for n.m.r. by dissolving them in $D_2O$ (99.7 %) after freeze-drying three times from $D_2O$ solutions.

1-Dimensional (1-D) $^1H$ and $^{13}C$ n.m.r. spectra were recorded at 20°C. $^1H$ and $^{13}C$ chemical shifts were measured with reference to internal acetone, $\delta$ H 2.21 and $\delta$ C 31.0 p.p.m. downfield of tetramethylsilane or DSS. All 2-dimensional (2-D) n.m.r. experiments were performed at 25°C or 80°C using the APT[84], COSY[77] and HETCOR[78] techniques.

2.5.6. Uronic acid was determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen[85].

## 2.6 **ACID HYDROLYSES**

Hydrolyses of samples, in sealed borosilicate glass tubes, were carried out under nitrogen, in 2 M TFA at 100°C for 18 h. Removal of the volatile acid by freeze-drying of the hydrolysates was followed by derivatization for g.l.c. analyses. Neutral sugars were converted to alditol acetates by the method of Albersheim et al[80], and also the method of Blakeney et al[86]. Any polyols present were acetylated with the alditols from sugars and simultaneously analysed. Results were corrected for degradation of proportions of the sugar residues during the hydrolyses[22,87,88].

## 2.7 METHYLATIONS AND RELATED EXPERIMENTS

### 2.7.1 Methods of Methylation

Methylation of samples was performed by the methods of Hakomori[67] (as modified by Phillips and Fraser)[68], Purdie[66] and Isogai et al[89].

#### 2.7.1.1 The method of Hakomori

Polysaccharides or oligosaccharides containing uronic acid were first decationised with Amberlite IR-120($H^+$)resin and freeze-dried. The sample was then vacuum-dried over $P_2O_5$ at 40° C for at least 16 h before being dissolved in dry DMSO. Methylation was performed under $N_2$ in a round-bottomed flask sealed with a serum cap. The base potassium methylsulphinylmethanide (potassium dimsyl; 2 M[68]), prepared by the addition of dry DMSO to dry KH at 0°, was added to the polysaccharide solution so that an excess of 40% over the number of equivalents of hydroxyl plus carboxyl was present. Contact time with the base ranged for 1 to 4 hours, the presence of excess base being confirmed by the removal of a drop of the reaction mixture for testing with triphenylmethane[68]. The solution of alkoxide, frozen in ice before the addition of methyl or trideuteriomethyl iodide, was stirred at room temperature for at least 1 hour after

this addition. Polysaccharides were recovered by extraction with a water-chloroform mixture (1:3 ; 2 vols), followed by dialysis of the chloroform extract. The non-dialysable mixture was extracted with more chloroform which was then removed by evaporation, and the products were purified by chromatography on a Sephadex LH-20 column, eluted with ethanol-chloroform (2:1 v/v), the passage of carbohydrate being monitored by the anthrone-sulphuric acid reagent[90]. Methylated oligosacharides were isolated from the reaction mixture by extraction with chloroform and then removal of salts and DMSO with water. Where necessary the products were purified by Sephadex LH-20 chromatography, the completeness of methylation being monitored by infrared spectroscopy, where the presence of O-H stretch at 3400cm$^{-1}$ would indicate the need for further methylation by the method of Purdie.

### 2.7.1.2 **The method of Purdie**

The dried partially-methylated poly- or oligosaccharide was dissolved in methyl iodide and refluxed (with the exclusion of atmospheric moisture) in the presence of $Ag_2O$ until methylation was completed. The procedure usually necessitated several additions of fresh reagents, depending on the difficulty of methylation. The methyl iodide was removed under reduced pressure after the AgI formed had been removed by filtration through Celite 545. The product was

dried at 40°C and the treatment above repeated as many times as needed. The degree of methylation was again monitored by infrared spectroscopy.

### 2.7.1.3 **The method of Isogai**

The dried polysaccharide (50 mg) was dissolved in DMSO (4.4 mL), heated in a round-bottomed flask under $N_2$ at 60°C for 0.5 h, cooled to room temperature, and stirred until a clear solution was obtained. Dry powdered NaOH (0.6 g) was added and stirring was continued for at least 1 h after which time the mixture was frozen. Methyl iodide (2.4 mL) was added and stirring was continued at room temperature for 1 h. The solution was gradually warmed to 60°C over a period of 0.5 h and maintained at this temperature for at least 1 h. The product was isolated by dialysis or chloroform extraction, depending on its molecular weight.

If the polysaccharide was not readily soluble in DMSO, then 0.2 mL of a $SO_2$-DMSO (0.3 g/mL) solution was added followed by 0.1 mL of diethylamine and stirring was continued until a clear solution was obtained. The solid base was then added.

This method provided a facile one-step methylation of oligosacharides, avoiding the disadvantages of the Hakomori method such as possible depolymerization, the limited stability of the reagent, the dangers of working with KH and the necessity for more than one methylation procedure.

In all cases the permethylated polysaccharide was hydrolysed in 2 M TFA at 100°C for 18h and the TFA was removed by freeze-drying or co-evaporation with water under reduced pressure. The hydrolysates, dissolved in methanol, were examined by p.c. (solvent A) using the mixture of partially methylated sugars from hydrolysis of methylated Virgilia oroboides gum[43] and other authentic specimens mentioned in the text as standards. The methylated aldoses were derivatised to p.m.a.a.s for analysis by g.l.c. and g.l.c.-m.s.

In methylation analyses, effective carbon response factors[81] were used in quantification.

### 2.7.2 **Lithium Aluminium Deuteride (LAD) Reduction**

The methyl-esterified carboxyl groups were reduced by the method of Aman et al.[91]. Lithium aluminium deuteride (LAD) was heated for 30 minutes at 80°C in dry THF in a Reactivial followed by centrifugation of the suspension, and the required proportion of this was added to the solution of the

methylated poly- or oligosaccharide in dry THF. The resultant solution, in a Reacti-vial, was heated at 80°C for 18h. Moist ethyl acetate was then added to the reduced product to decompose the excess LAD. The suspension was filtered through Celite 545 and the filter pack was washed with warm $CHCl_3$. All the washings were pooled with the filtrate, evaporated to dryness under reduced pressure and were finally dried at 40°C under vacuum. The absence of carbonyl stretches on infrared spectroscopy was assumed to be indicative of completeness of reduction of the carboxyl groups. A further check was the disappearance of the pink spot corresponding to uronic acid, which was given by the p-anisidine HCl spray on p.c. (solvent A) of the hydrolysate of the methylated polysaccharide.

## 2.8 DEGRADATION OF URONATE ESTER RESIDUES

### 2.8.1 Potassium Dimsyl Method[56,68]

Samples of the methylated products (20 mg) together with a trace of toluene-p-sulphonic acid were dissolved in a mixture of DMSO and 2,2-dimethoxypropane (19:1; 3 mL) and stirred with potassium methylsulphinylmethanide (1.5 M; 2 mL) for 18 h. After cooling in ice water methyl iodide (10 mL) was added and the solutions were stirred for at least one hour. The solutions were then diluted with water and dialysed against running tap water for 48 h before

being extracted with chloroform. In the case of oligosaccharides, where dialysis was not possible, the product was extracted from the solution with chloroform. Evaporation of chloroform gave the degraded methylated product in good yield.

Samples of degraded polysaccharides or oligosaccharides were hydrolysed in 2 M TFA at 100°C for 18 h and the sugars present in the hydrolysates were derivatised to alditol acetates and analysed by g.l.c. and g.l.c.-m.s.

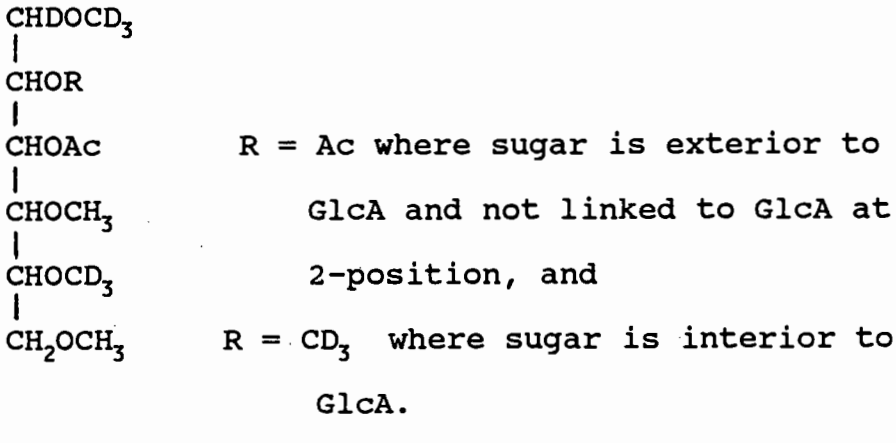
### 2.8.2 **DBU (Non-Nucleophilic base) Method**[20,33,40]

The methylated polysaccharide (25 mg) was dissolved in benzene (4 mL) in a glass tube, and 1,5-diazabicyclo (5.4.0.) undec-5-ene (DBU; 2 mL) and acetic anhydride (1 mL) were added. The tube was sealed and heated at 100° for 18 h. The reaction product was washed with M HCl and the benzene layer was washed with water, dried, and concentrated. The residue was evaporated several times with methanol, then methanolic sodium methoxide (4 mL) was added dropwise until a basic pH was maintained. The mixture was allowed to stand for 5 h, sodium borodeuteride (150 mg) was added and the mixture was allowed to stand overnight. Acidification with acetic acid was followed by evaporation (3 times) with

methanol. The degraded product was extracted with a 1:4 (v/v) chloroform-water, the aqueous phase being tested with anthrone to ensure that no carbohydrate was present. The chloroform phase was evaporated and the residue was hydrolysed with 10% acetic acid at 100°C for 1 h, freeze-dried and then extracted with chloroform. After removal of the chloroform under reduced pressure, the product was deuteriomethylated by adding potassium dimsyl (2 M), and $CD_3I$ (2 mL) and stirring for 3 h. The product was extracted with chloroform, and the extract was washed with water and evaporated to dryness. The residue was hydrolysed with 2 M TFA at 100°C for 6 h. After removal of TFA, sodium borohydride was added and the mixture was allowed to stand overnight. The excess $NaBH_4$ was neutralised with acetic acid, and, after evaporation, borate was removed from the residue by evaporation with methanol and the alditols were acetylated with acetic anhydride. The resulting alditol acetates were analysed by g.l.c. and g.l.c.-m.s.

This method of base degradation of esterified and methylated polysaccharide allowed the isolation of fragments both exterior and interior to the points of cleavage of the esterified GlcA units. Sugars detached by β-elimination from positions exterior to the acidic residues were labelled with deuterium at C-1 before removal, by mild acid hydrolysis, of the unsaturated acidic components remaining attached to the interior sugar units. In this sequence the

alditol formed by borodeuteride reduction was subsequently further labelled by deuteriomethylation at positions 1 and 5 of the ring-opened exterior sugar unit, and, if the parent sugar residue was located interior to acid, at the position to which the acid had been joined (possibly the C-2 position in **2** below). The resulting derivative then had the following structure (**2**):




**2**

# 3. INVESTIGATION OF THE POLYSACCHARIDE EXUDATE FROM *Hakea sericea* (i) DISCUSSION

## 3.1 INTRODUCTION

*Hakea sericea*, also known as silky Hakea (fam. *Proteaceae*), is a species which was introduced into the Republic of South Africa from Australia during the past century. It was used in the Cape Flats as a sand stabiliser and now occurs widely throughout the region from Cape Town to Grahamstown and up to 100 km inland. Clumps of trees have been noted as far afield as Natal, but by far the largest number occur with the mountainous fynbos vegetation and on the sandstone and granite areas of the mountain ranges, such as the Constantiaberg and Hottentots Holland mountain range in the Cape, which have predominantly winter rainfall.

The typical tree grows up to 2 to 3 metres high, occasionally up to 5 metres with a single trunk which is multi-branched. The young branches are covered with short, fine, silky hairs which disappear on maturity. The dark green leaves are smooth and needle-shaped, up to 40 mm long, 1 mm in diameter and are characteristically sharp-pointed. From June to December cream- coloured flowers appear, which give the plants a silver glow in misty conditions. The fruit is an extremely hard achene and is the size of an almond nut; each contains

two-winged seeds. These plants have proliferated to such an extent that they are now classified as pests throughout South Africa. A photograph is presented below.

## 3.2 ORIGIN AND ISOLATION

The sample of H.sericea gum exudate used in the present work was collected near Grahamstown from a tree that was identified as H.sericea by the Bolus Herbarium, UCT. The gum, when stirred in water overnight (2% w/v; 25°C), afforded two polysaccharide products, one readily soluble in water (HSWS) and the other a less soluble, gel-like fraction (HSLS). The latter fraction dissolved easily and almost immediately when heated to 50°C (2% w/v concentration). Freeze drying of these two fractions yielded HSWS as a white fluffy product (150 g; 60% by weight) and HSLS as a slightly coarse white fraction (100 g; 40% by weight). An analytical sample of each was prepared by precipitation with absolute ethanol (5 vols) from an aqueous solution (5 g in 25 mL water). No ethanol-soluble carbohydrate was present. The precipitated product, collected by freeze-drying in the usual manner, was characterised (see Table 1).

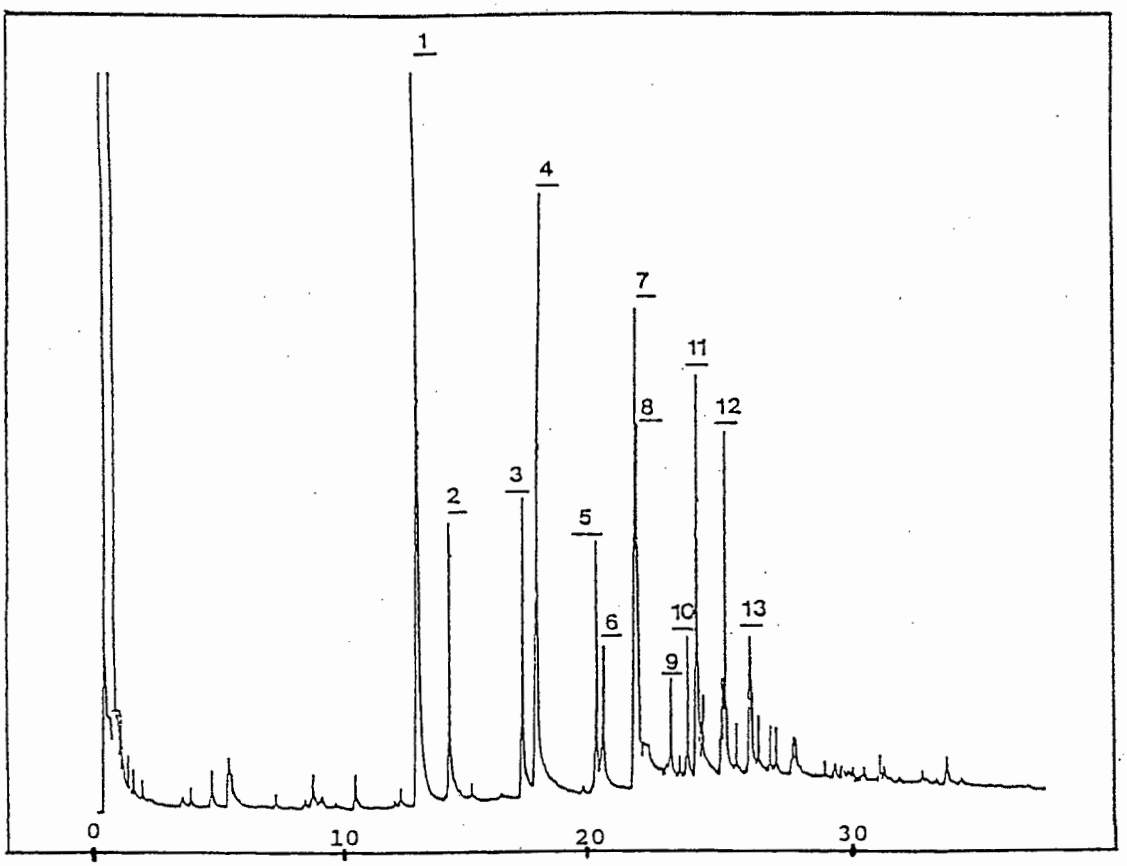
TABLE 1. PROPERTIES OF *Hakea sericea* EXUDATE, WATER-SOLUBLE (HSWS) AND *Hakea sericea* LESS-SOLUBLE (HSLS) FRACTIONS

| Properties | *H.sericea* exudate | |
|---|---|---|
| | HSWS | HSLS |
| Water solubility | Total | Variable |
| $[\alpha]_D$ | +0.4° (c 1.0) | +0.2° (c 1.0) |
| Microanalysis: | | |
| C (%) | 42.8 | 42.8 |
| H (%) | 5.8 | 5.7 |
| N (%) | - | - |
| Sugar proportions[a] (mol%) | | |
| Arabinose | 20 | 20 |
| Xylose | 7 | 7 |
| Mannose | 7 | 7 |
| Galactose | 59 | 59 |
| Glucuronic acid[b] | 7 | 7 |
| $\bar{M}_w$[c] | $>2.10^6$[d] | $>2.10^6$ |
| Acid equivalent[e] | 2400 | 2400 |
| Periodate consumption[f] (mmol $g^{-1}$) | 6.2 | 6.8 |

a Proportions of neutral sugars by g.l.c. analysis of the hydrolysate as alditol acetates.
b Determined by the colorimetric method of Blumenkrantz *et al.*[87] ; figures quoted for the anhydride form of glucuronic acid.
c From steric-exclusion chromatography.
d Single peak on Sepharose 4B.
e Determined by titration with base.
f Determined by the arsenite titration method[65].

## 3.3 METHYLATION AND RELATED ANALYSES OF H. sericea WATER-SOLUBLE FRACTION (HSWS)

The ease with which methylation is achieved depends upon the structure and size of the poly- or oligosaccharide. Per-O-methylation was achieved in this study by an initial treatment using the modified Hakomori method[67,68], followed by further methylation of the partially methylated product by the method of Purdie[66] (usually 3 successive treatments). At this point completeness of methylation was confirmed by the absence of HO- stretches (3200 to 3500 $cm^{-1}$) in the IR spectrum of the fully methylated product. Also useful was the presence of the large absorption band at ~1100 $cm^{-1}$ due to $-O-CH_3$ groups, and the band at ~1730 $cm^{-1}$ resulting from the presence of carbonyl groups in esterified uronic acid residues. The nature of the GlcA linkages was ascertained by lithium aluminium deuteride (LAD) reduction of the carboxylate ester function in the permethylated polysaccharide to form a $CD_2OH$ group which has m/z 2 units higher than that of a $CH_2OH$ and is therefore distinguishable by g.l.c.-m.s. The methylated product was hydrolysed, converted to partially methylated alditol acetates and submitted to g.l.c. and g.l.c.-m.s. analysis (Fig. 1).




Key: 1 = 2,3,5-Ara<u>f</u>, 2 = 2,3,4-Xyl, 3 = 2,3-Ara<u>p</u>, 4 = 2,3,4,6-Gal, 5 = 2,4,6-Gal, 6 = 2,3,6-Gal, 7 = 2,3,4-Gal, 8 = 4,6-Man, 9 = 2,6-Gal, 10 = 2,3-Gal, 11 = 2,4-Gal, 12 = 2-Gal, 13 = 3-Gal

FIGURE 1. G.L.C. TRACE OF THE PARTIALLY METHYLATED ALDITOL ACETATES DERIVED FROM HSWS

TABLE 2. METHYLATION ANALYSIS DATA FOR HSWS, HSLS AND LAD-REDUCED METHYLATED SAMPLES OF HSWS AND HSLS

| PARTIALLY METHYLATED SUGAR (AS ALDITOL ACETATES[a]) | LINKAGE MODES | METHYLATED | | LAD-REDUCED METHYLATED | |
|---|---|---|---|---|---|
| | | HSWS (mol%) | HSLS (mol%) | HSWS (mol%) | HSLS (mol%) |
| 2,3,5-Ara | T-Araf | 16 | 15 | 19 | 16 |
| 2,5-Araf | -3)-Araf | 2 | 2 | tr | 2 |
| 2,3-Ara | -4)-Arap or -5)-Araf | 6 | 5 | 3 | 5 |
| 2,3,4-Xyl | T-Xylp | 6 | 6 | 8 | 5 |
| 2,3,4,6-Gal | T-Galp | 15 | 16 | 18 | 20 |
| 2,4,6-Gal | -3)-Galp | 8 | 9 | 12 | 8 |
| 2,3,6-Gal | -4)-Galp | 2 | 2 | 3 | 2 |
| 2,3,4-Gal | -6)-Galp | 17 | 18 | 16 | 17 |
| 2,3-Gal | -4,6)-Galp | 1 | tr | 1 | tr |
| 2,6-Gal | -3,4)-Galp | tr | tr | tr | tr |
| 3,4-Gal | -2,6)-Galp | tr | tr | 9 | 2 |
| 2,4-Gal | -3,6)-Galp | 8 | 9 | tr | 8 |
| 3,6-Gal | -2,4)-Galp | tr | tr | tr | tr |
| 2-Gal | -3,4,6)-Galp | 4 | 4 | 2 | 4 |
| 3-Gal | -2,4,6)-Galp | 1 | tr | tr | tr |
| 4-Man | -2,3,6)-Manp | tr | tr | tr | tr |
| 4,6-Man | -2,3)-Manp | 7 | 7 | 5 | 6 |
| 2,3-Glc($d_2$) | -4)-GlcpA | - | - | 1 | 2 |
| 2-Glc($d_2$) | -3,4)-GlcpA | - | - | 3 | 3 |

For the Gal and GlcA units, there is a possibility that the linkages listed as -4 for p residues could be -5 for f residues; this, however is unlikely

a 2,3,4,6-Gal = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol, etc

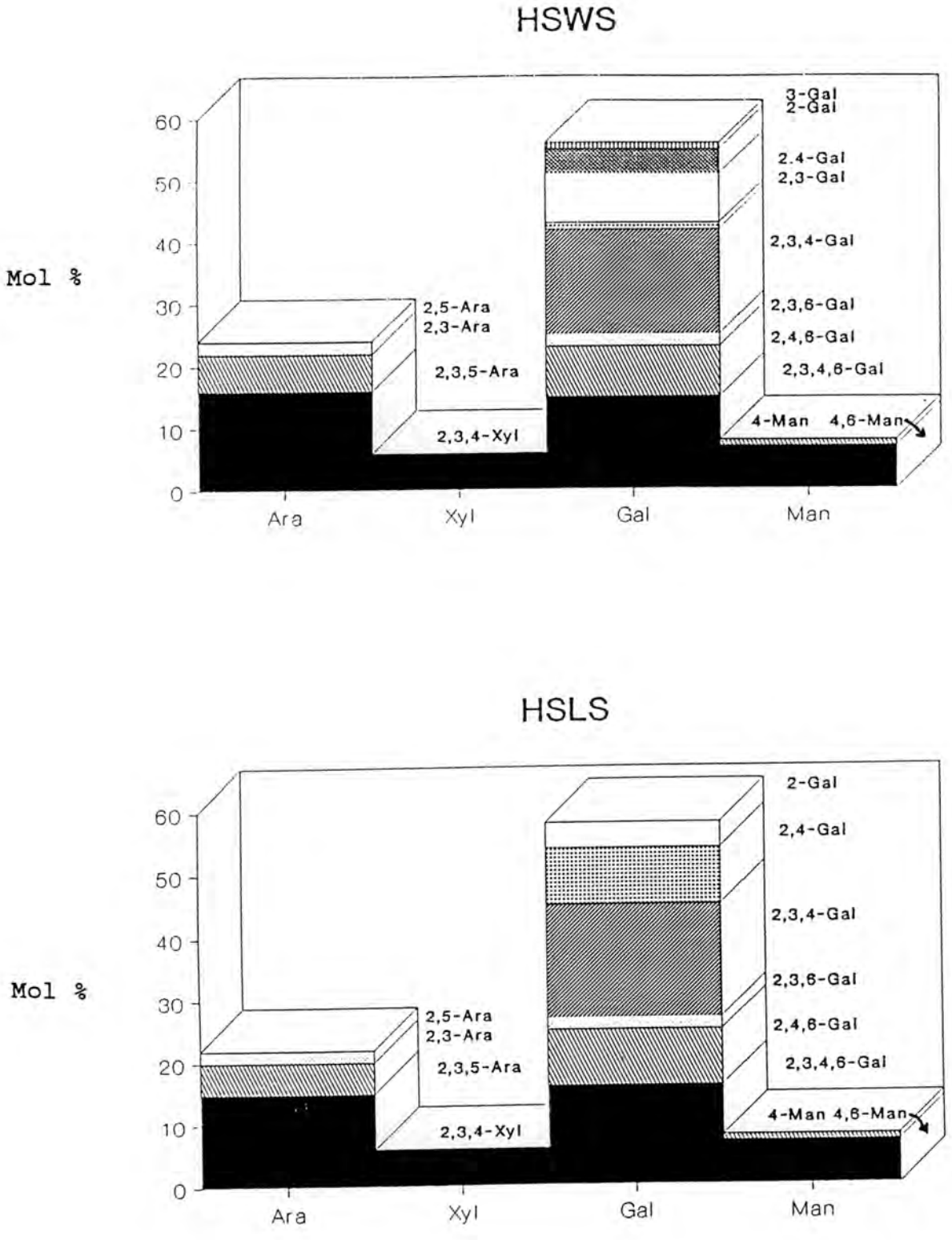



FIGURE 2. HISTOGRAMS OF METHYL ETHERS IN HSWS AND HSLS

The results are presented in Table 2 and as a histogram in Fig. 2. The main conclusions are as follows:

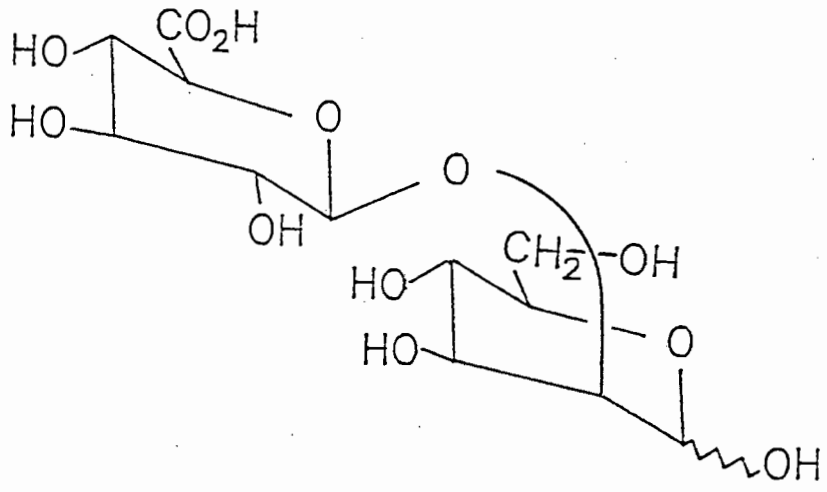
Arabinose: The bulk of the arabinose occurred terminally and in the furanose form with the presence of some 2,3-di-*O*-Me-Ara indicating the occurrence of (1-4)-linked Ara*p* or (1-5)-linked Ara*f* (*ca*. 25% of total Ara). Also present was a trace of 2,5-di-*O*-Me-Ara, further emphasising the existence of most, if not all, of the Ara in the furanose form. This is further corroborated by complete removal of all the Ara by mild hydrolysis with dilute acid (see Section 3.4.5). The existence of chains of Ara, apparently unbranched, is indicated by the methylation results.

Xylose: All xylose was present as end-group in the pyranosyl form, most of which was resistant to partial hydrolysis by dilute acid (Section 3.4.5).

Mannose: The mannose was present mainly as 2,3-linked units with a trace of 4-*O*-Me-Man indicating some further substitution on O-6.

Glucuronic acid: LAD reduction of the methylated polysaccharide revealed the glucuronic acid to be 4-linked and 3,4-linked in a ratio of 1:3. As seen from Table 1 the GlcA and Man were present in a 1:1 ratio which could infer a

close relationship between these sugars in the structure. In a previous study of the polysaccharide gum from Hakea acicularis[45] (H. sericea) the aldobiouronic acid 2-O-( β -D-glucopyranosyluronic acid)-D-mannose (3) was isolated in significant amount.



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Galactose: This sugar was present in many linkage types and this methylation analysis indicates that all galactose exists only in the pyranose form. The end-group and 6-linked forms are present in approximately equal proportions with lesser quantities of 3-linked and 3,6-linked forms (in the ratio 16:17:8:8). Also present, albeit in trace amounts, were 3,4- , 2,6- , 2,4- , 3,4,6- and 2,4,6-linked galactosyl units, indicating the possibility of a highly branched galactan network.

## 3.4 SMITH DEGRADATION OF *Hakea sericea* GUM

### 3.4.1 Sequential Smith Degradation of HSWS

#### 3.4.1.1 First Smith degradation

Since methylation analysis of HSWS showed the presence of a high proportion of periodate-susceptible residues (64%), Smith degradation was performed on a large sample of HSWS (Scheme 1), with the objective of isolating the polysaccharide core and possibly revealing structural regularity.

Periodate oxidation of HSWS followed by reduction, mild hydrolysis and fractionation with methanol-acetone yielded two products: a high molecular weight insoluble fraction (HS-SD1; 43% w/w) and a soluble fraction (HS-SD1 solubles; 21% w/w) composed mainly of glycerol and arabinose (5:1), with a trace of xylose. The product, HS-SD1 eluted from the Sepharose 4B column in a single peak at $\bar{M}_w$ 225 000 (Fig. 3.b). The decrease in $\bar{M}_w$ on Smith degradation indicated a few breaks at periodate-vulnerable site(s) within the polysaccharide structure, in addition to removal of peripheral sugars. This implies the presence of periodate-vulnerable sugar(s) joining adjacent periodate-resistant units each with an average molecular weight of 225 000.

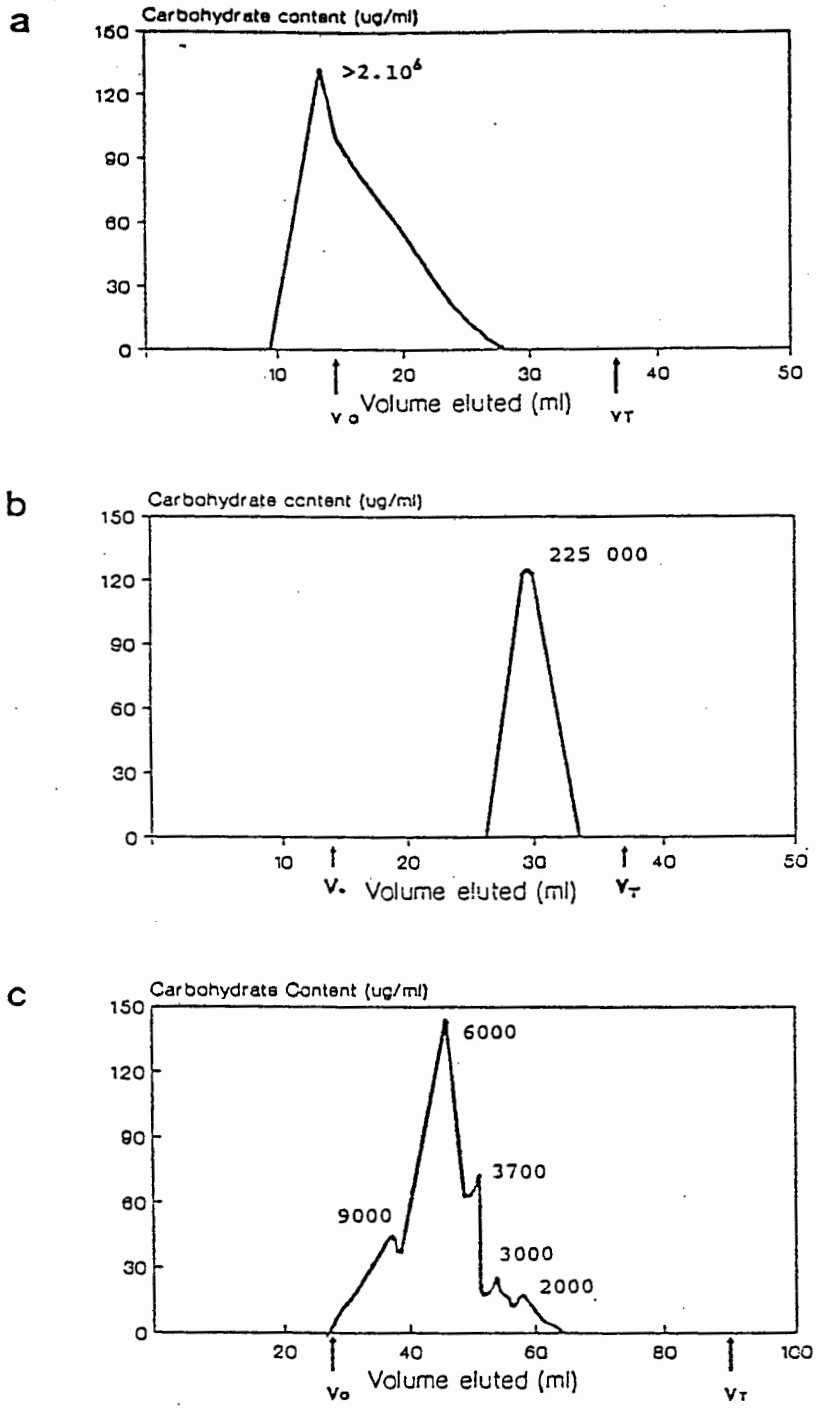



FIGURE 3. STERIC-EXCLUSION CHROMATOGRAPHY OF a. HSWS (SEPHAROSE 4B), b. HS-SD1 (SEPHAROSE 4B), and c. HS-SD2 (BIO-GEL P-10)

Analysis of component sugars in the HS-SD1 product (Table 3) indicated a drop in arabinose and xylose, with localisation or concentration of GlcA and Man (ratio 1:1) hinting at the presence of a structure having these two sugars closely linked, possibly in the form of the aldobiouronic acid, GlcA-2Man (the monomer)[45]. This monomer unit may occur singly, in oligomeric forms of different lengths[8,37], or with periodate-vulnerable sugar(s) interspersed between the monomers or oligomers. GlcA-2Man was shown to be present on p.c. (solvent C) of the hydrolysed HS-SD1 product prior to conversion to alditol acetates for g.l.c.

Methylation data for the methanol-acetone insoluble fraction showed the presence of Araf solely as end-group, remaining after cleavage and removal of the susceptible residues in the arabinose chains, such as 5-linked Araf and 4-linked Arap, or other periodate-vulnerable residues to which it may have been attached. Major residues present in enhanced proportion in HS-SD1 were 3-linked Gal, 6-linked Gal, and terminal Gal (ratio approx 1:1:1), whereas the presence of 2,4-di-O-Me-Gal in diminished proportion suggested that the linkage of periodate-vulnerable substituents such as terminal Araf or Xylp was to the O-6 of the otherwise 3-linked Galp, or to the O-3 of the otherwise 6-linked Gal.

TABLE 3. PROPERTIES OF SMITH DEGRADATION PRODUCTS FROM HSWS

| PROPERTIES | SD1 | SD2 | SD3 |
|---|---|---|---|
| Starting mass (g) | 15 | 5 | 0.5 |
| Product mass ratio of insolubles: solubles | 6.5:3.2 | 3.0:1.5 | 0.09:0.10 |
| Yield (%) | 43 | 58 | 18 |
| Periodate consumption of starting material (mmol $g^{-1}$) | 4.0 | 6.0 | 6.0 |
| INSOLUBLE PRODUCT: | | | |
| $[\alpha]_D$ | + 26.7° | + 72° | + 40° |
| $\bar{M}_w$ | 225 000 | 6 000 (4 200)[a] | 1 800 (1 500)[a] |
| Glycolaldehyde[b] ( %) | - | 1.0 | 4.2 |
| Acid equivalent | 1 080 | 800 | 2 425 |
| Sugar analysis (mol %): | | | |
| Erythritol | - | - | 19 |
| Ara | 9 | - | - |
| Xyl | 5 | - | - |
| Man | 22 | 22 | 20 |
| Gal | 45 | 53 | 49 |
| GlcA[c] | 19 | 24 | 8 |
| Methylation data (mol %): | | | |
| 2,3,5-Ara | 6 | - | - |
| 2,3,4-Xyl | 3 | - | - |
| 2,3,4,6-Man | tr | tr | tr |
| 2,3,4,6-Gal | 22 | 19 [19][d] | 34 |
| 3,4,6-Gal | - | - | - |
| 4,6-Man | 8 | 15 [4] | 22 |
| 2,4,6-Man | tr | tr [7] | - |
| 3,4,6-Man | - | - [3] | - |
| 2,4,6-Gal | 26 | 32 [17] | 22 |
| 2,3,4-Gal | 18 | - [7] | - |
| 2,3,6-Gal | - | - [3] | - |
| 2,4-Gal | tr | 6 [5] | 12 |
| 2,3-Gal | - | - [6] | - |
| 2-Gal | tr | 3 [3] | tr |
| 4(3)-Hex | tr | tr [tr] | tr |
| 2,3-Glc ($d_2$)[e] | tr | 22 [-] | 8 |
| 2-Glc ($d_2$)[e] | 18 | 3 [-] | - |

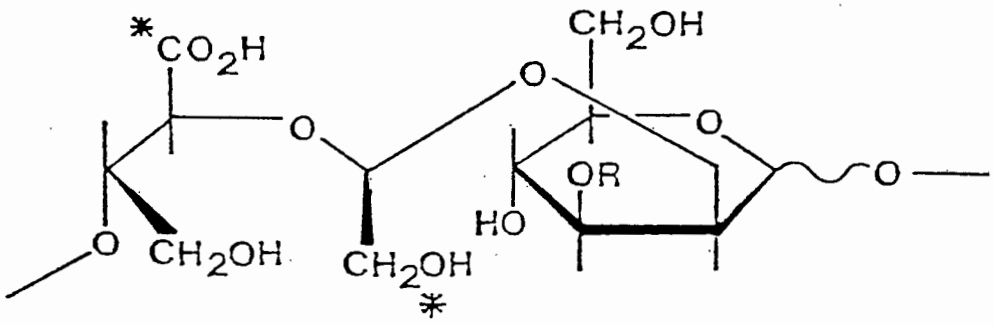
a Figures in parentheses indicates the $\bar{M}_w$ at the second limit
b Determined colorimetrically by method of Dische[93]
c Determined colorimetrically by method of Blumenkrantz[87]
d Figures in parentheses, [ ] are for dimsyl-treated HS-SD2
e Determined after LAD reduction[91]

Furthermore, increase in 6-linked Galp would result from removal of oxidizable substituents from the O-3 of 3,6-linked and also from O-3 and O-4 of the 3,4,6-linked branched Galp; similarly the increase in 3-linked Galp would be due to removal of the oxidizable residues from O-4 or O-6 of 3-linked, branched Galp. Importantly, most of the mannose was detected on methylation analysis as the 4,6-di-O-methyl derivative i.e. in 2,3-linked form, with a trace of terminal group and 3-linked mannose, while LAD reduction revealed the glucuronic acid to be mostly 3,4-linked with a trace amount of the 4-linked form. This strongly suggested some significant strucural involvement of the -4GlcA-2Man- aldobiouronic acid unit substituted at O-3 of Man and mainly at O-3 of the GlcA by galactose. Thus in SD1 the aldobiouronic acid could have a large envelope of sugar units around it, in which the substituents are in chains terminating occasionally in Araf and Xylp but mainly in Galp (Structure **6**). The fact that free glycerol is present in the low molecular weight SD1 soluble product is evidence of the degradation of formerly terminal hexopyranosyl and pentofuranosyl groups viz. Galp, Xylp and Araf, as well as 6-linked Galp.

### 3.4.1.2 **Second Smith degradation**

Since the high molecular weight HS-SD1 product was composed of a significant proportion of periodate-susceptible residues (59%), it was decided to subject this product to a further SD treatment. The periodate consumption value showed a 9% over-oxidation of the HS-SD1 which was not considered to be excessive. Barium acetate was added to remove the excess periodate and so avoid dialysis which could result in the loss of any low molecular-weight material generated. Otherwise the procedure followed (Scheme 2) was similar to that which gave the HS-SD1 products. The methanol-acetone insoluble portion of the HS-SD2 product, $\bar{M}_w$ 6000 (polydisperse; 50% yield; Table 3; Fig. 3.c) and low molecular weight soluble product (30% yield) were isolated, the latter being composed of glycerol (10+), arabinose (+), xylose (+), and erythritol (trace).

The HS-SD2 product was shown to contain 1% bound glycolaldehyde which could be part of an acetal link between a unit ending in degraded 4-linked GlcpA, now in the form of bound erythronic acid (see later, Fig. 8), and the adjoining periodate-resistant unit[28].



4 *

* Lactonization could occur between starred groups

The difficulty in severing the carbohydrate chains by normal acid treatment at points where glucuronic acid units had been opened by the oxidation-reduction procedure was also noted in similar experiments on gum ghatti (from Anogeissus latifolia)[20], and on apricot-tree gum[28] (from Prunus armeniaca), and Grevillea robusta gum[33]. It was thought to be the result of lactonization between certain functional groups (Structure 4) forming a glycoside-like structure linking the periodate-resistant units.

The erythronic acid-glycolaldehyde acetal system that essentially links periodate-resistant units to each other, thus increasing the $\bar{M}_w$, is destroyed very slowly by exposure to M TFA at room temperature, so that the usual period of a few days is insufficient. Thus prolonged contact with M TFA, for 42 days, was employed to cleave all these acetals and resulted in a periodate-resistant product (HS-SD2-42) with a lower $\bar{M}_w$ of 4200 (polydisperse; Fig. 4.a).

This product, regarded as the second limit of depolymerization, had the same proportions of neutral sugars as the SD2, viz. Man 20%, GlcA 25% and Gal 55%.

Methylation analyses, LAD reduction and potassium dimsyl degradation of HS-SD2 revealed the following important structural information:

(i) The predominant components present were 3-linked galactose and terminal galactose in ratio 3:2, less 2,3-linked mannose and 4-linked GlcpA (in the ratio 0.7:1), with smaller fractions of 3,4,6-linked and 3,6-linked galactose and 3,4-linked GlcA as branch points, and some 4,6-linked Gal.

(ii) The mannose and glucuronic acid residues were again localised in the product, but differently; the Man was present only as 2,3-linked residues, and in a yield lower

than expected, probably due to the resistance to hydrolysis of the glycosidic bond between the GlcA and the mannose of the aldobiouronic moiety.

(iii) Potassium-dimsyl base degradation resulted in the production of 2,3,4,6-tetra-O-methylmannose and an increase in 2,4,6-tri-O-methylmannose of 6%, with 4% of 4,6-di-O-methylmannose remaining intact. These were both labelled at O-2 with **D**, indicating that at least some of the degraded GlcA had been joined to the O-2 of Man. This was also inferred from the concomitant decrease in 2,3-linked Man.

### 3.4.1.3. **Third Smith degradation**

Once again the presence of a significant proportion of periodate-oxidizable sugars, 32%, showed the necessity for a further Smith degradation. Two products were obtained, one, composed of glycerol (++) and Gal (+), soluble in the methanol-acetone mixture and the other insoluble in the mixture (SD3). The HS-SD3 product (see Table 3; Fig. 4.b) was shown to contain 4.2% glycolaldehyde which indicated the presence of ca. 1 mole per mole of oligosaccharide of $\bar{M}_w$ 1800 (with 2 major components at mol. wts. 2950 and 950 present in the ratio 1:1). As stated above the glycolaldehyde is part of the linkage between the oxidized GlcA units and protected Man residues as shown in structure **4**.

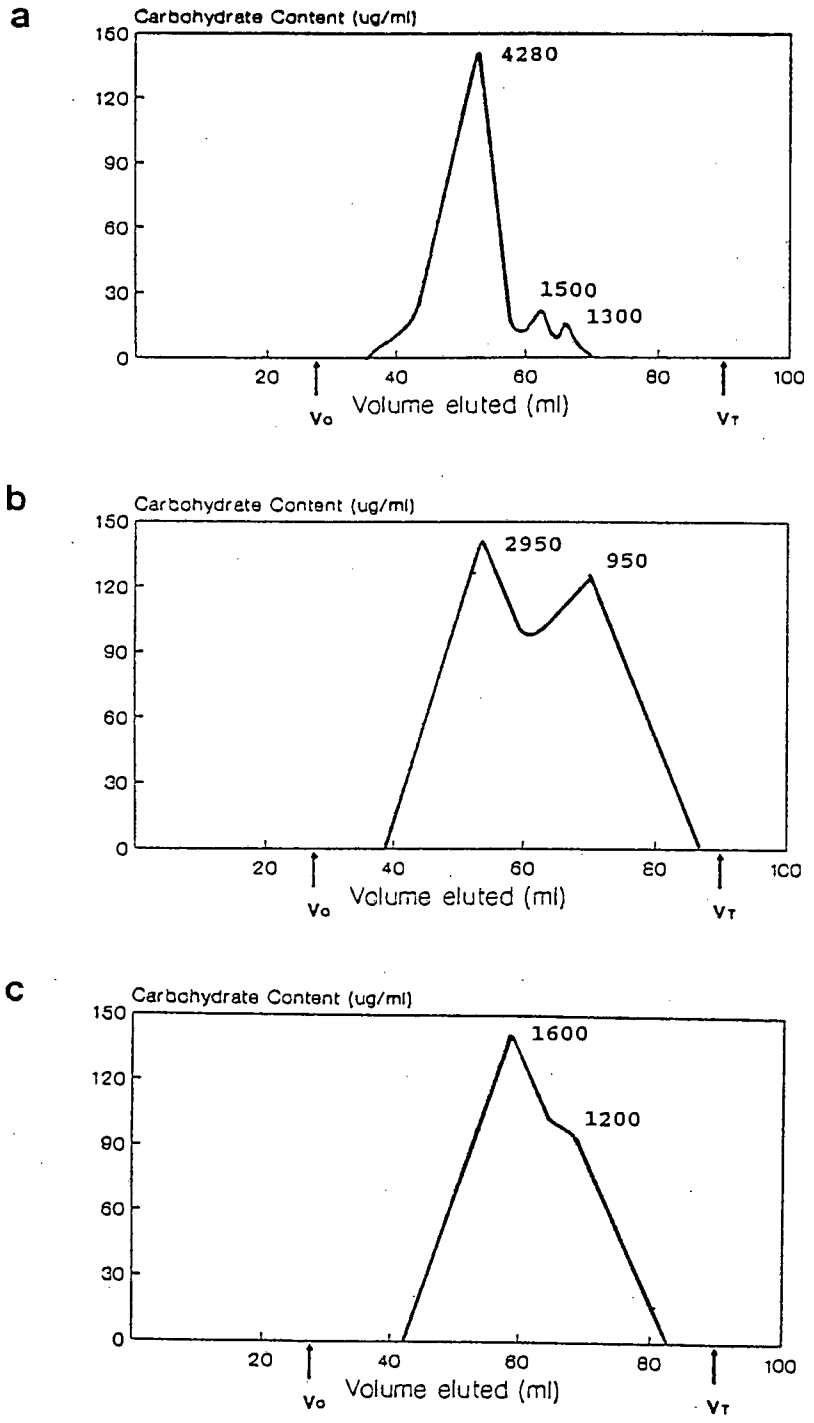
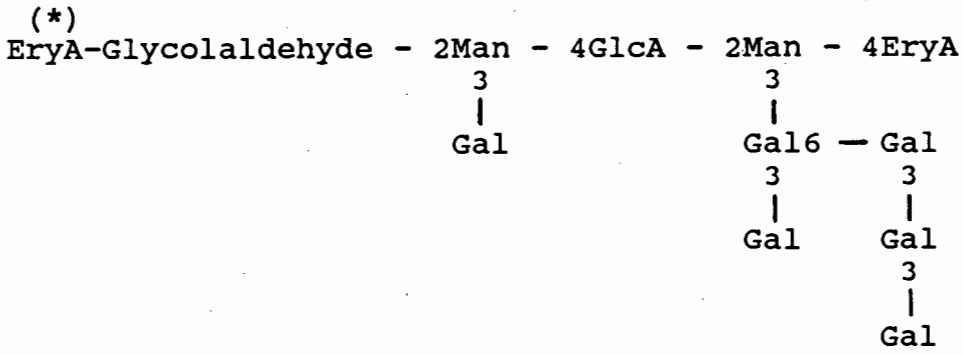



FIGURE 4. STERIC-EXCLUSION CHROMATOGRAPHY OF a. HS-SD2-42, b. HS-SD3, and c. HS-SD3-50 (ALL ON COLUMNS OF BIO-GEL P-10; THE SAMPLES ARE DESCRIBED IN THE TEXT).

These glycolaldehyde acetal units were cleaved from the SD product by hydrolysis after 50 days of exposure to M TFA. The product was freeze dried and was fractionated with methanol-acetone mixture (1:3) to yield two products. One was insoluble in the mixture, viz. HS-SD3-50, with $\bar{M}_w$ 1500 and contained two major components with mol. wts. 1600 and 1200 present in molar ratio ca 1:1 (Fig. 4.c), as well as containing 8% glucuronic acid. The other product soluble in the mixture, comprised Gal. The product HS-SD3-50 could be represented by a structure of 9 to 10 sugar units, in which one is GlcpA. Methylation analysis including LAD reduction showed the presence of major proportions of terminal Gal, 3-linked and 3,6-linked galactose, 2,3-linked mannose and 4-linked GlcA in a ratio ca 3:2:1:2:1.

The presence of erythritol (19%) in the hydrolysate of HS-SD3 is in keeping with the assertion that degraded 4-linked GlcpA yields erythronic acid which, after conversion to the lactone by acid treatment and reduction with $NaBH_4$, would yield erythritol, detected as its acetate by g.l.c. analysis of the alditol acetates derived from the hydrolysate. The two units of erythronic acid could be located one at each end of the molecule (**5**).

From the information stated above the periodate-resistant "core" unit (HS-SD3) could be represented by many structures of which **5** is one :

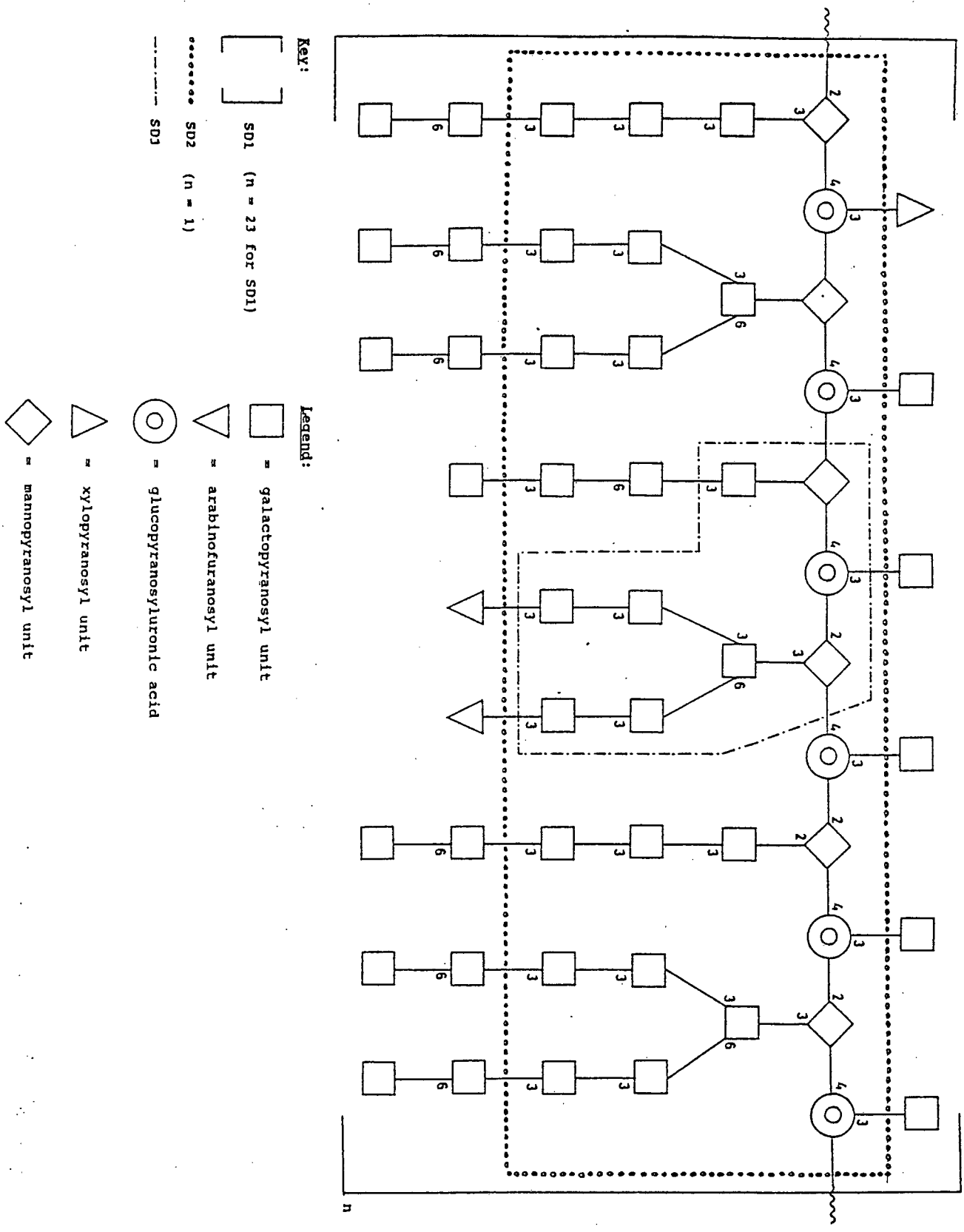



**5**

Thus the results obtained from sequential Smith degradation indicate that the innermost core of the polysaccharide must contain at least one aldobiouronic acid GlcpA-2Man moiety with a galactose side-chain attached to the 3-position of the mannose. The galactose units in the pendant side-chain include 3-linked, 3,6-linked and terminal groups (**5**). This assembly of nine sugars has attached to each side of it a periodate-susceptible sugar which in turn is linked to another one or more units such as **4**. If a glucuronomannoglycan type of structure is present in the polysaccharide then this susceptible sugar could be a deprotected 4-linked GlcA residue(s), shown as EryA (*) in **5**, which was previously in the SD1 product protected from periodate oxidation by substitution at O-3. This structural unit **5** would thus

be the core surrounded by a larger galactan network with xylose end-groups, arabinofuranosyl chains terminating in arabinose end groups, and Galp end-groups, differing from that of the branched 3,6-linked D-galactan framework typical of arabinogalactan-based plant gums. Utilising **5** as a core, a structure for the periodate-resistant unit isolated at the SD2 level would be **6**.

The methylation data obtained for this product are in accordance with this structure except with respect to the proportion of GlcA which is higher than that postulated in the model. However this could be offset partly by the slightly low proportion of T-Gal indicated by methylation analysis, which may be due to loss of 2,3,4,6-Gal during the concentration phase of alditol acetate production. If the T-Gal is increased to compensate for this, the GlcA content as shown by methylation analysis will decrease and approach that required by the model. Similarly the T-Gal "loss" could also account for some of the extra T-Gal which, along with the extra 3-linked Gal, is utilised in the SD1 model (**6**).




6

A PROPOSED STRUCTURE FOR H.sericea SD1 PRODUCT

### 3.4.2 **Partial Acid Hydrolysis (Autohydrolysis) of HSWS**

In an effort to gain more information about the structure of this polysaccharide it was decided to remove most if not all of the arabinofuranosyl and some xylopyranosyl end-groups, together with some of the terminal galactopyranosyl groups, from HSWS by partial hydrolysis with dilute sulphuric acid (Scheme 4). The water soluble product (Table 4) was then subjected to Smith-degradation analysis with a view to revealing the intact innermost core structure.

Partial hydrolysis yielded a product, A, of $M_w$ 150 000 (Fig. 5) devoid of arabinose (Table 4), and with the remaining xylose present as end-group.

The mannose content was slightly lower than that of the GlcA (ratio 0.8:1) and the concentration of these two residues in the product was evident. Methylation analysis (Table 5) showed Man to exist mainly as 2,3-linked residues with traces of 2-linked and end-group Man. GlcA was 3,4-linked and 4-linked (ratio approximately 1:2), and the major sugar constituent, galactose, was present as end-group, 6-linked and 3-linked residues. The suitability of a Smith degradation procedure was indicated by the high proportion (55%) of periodate-susceptible sugar residues.

TABLE 4. PROPERTIES OF AUTOHYDROLYSED HSWS (A) AND AUTOHYDROLYSED HSLS (A-LS)

| PROPERTY | A | A-LS |
|---|---|---|
| $[\alpha]_D$ | + 3° (c 1%) | + 44° (c 1%) |
| Microanalysis: | | |
| C (%) | 42.5 | 42.3 |
| H (%) | 5.8 | 5.8 |
| N (%) | - | - |
| $M_w$[a] | 150 000 monodisperse | 900 000 |
| Sugars (mol %) | | |
| Ara | tr | tr |
| Xyl | 7 | 8 |
| Man | 20 | 8 |
| Gal | 48 | 61 |
| GlcA[b] | 25 | 22.5 |
| Acid equivalent | 776 | 860 |

a Determined on column of Sepharose 4B
b Calculated as anhydride form.

TABLE 5. METHYLATION ANALYSIS OF A AND A-LS (COMPONENTS IDENTIFIED AS ALDITOL ACETATES)

| P.m.a.a. | A (mol %)[a] | A-LS (mol %)[a] |
|---|---|---|
| 2,3,4-Xyl | 6 | -[c] |
| 2,3,4,6-Man | tr | tr |
| 3,4,6-Man | tr | 3 |
| 4,6-Man | 17 | 16 |
| 2,3,4,6-Gal | 21 | 19 |
| 2,4,6-Gal | 2 | 8 |
| 2,3,6-Gal | 3 | - |
| 2,3,4-Gal | 24 | 24 |
| 2,3-Gal | tr | - |
| 2,4-Gal | 1 | 4 |
| 2-Gal | tr | 3 |
| 4(3)-Hex | tr | tr |
| 2,3-Glc ($d_2$)[b] | 18 | 9 |
| 2-Glc ($d_2$)[b] | 7 | 13.5 |

a Figures corrected for acid
b Determined after LAD reduction of the methylated A
c none detected

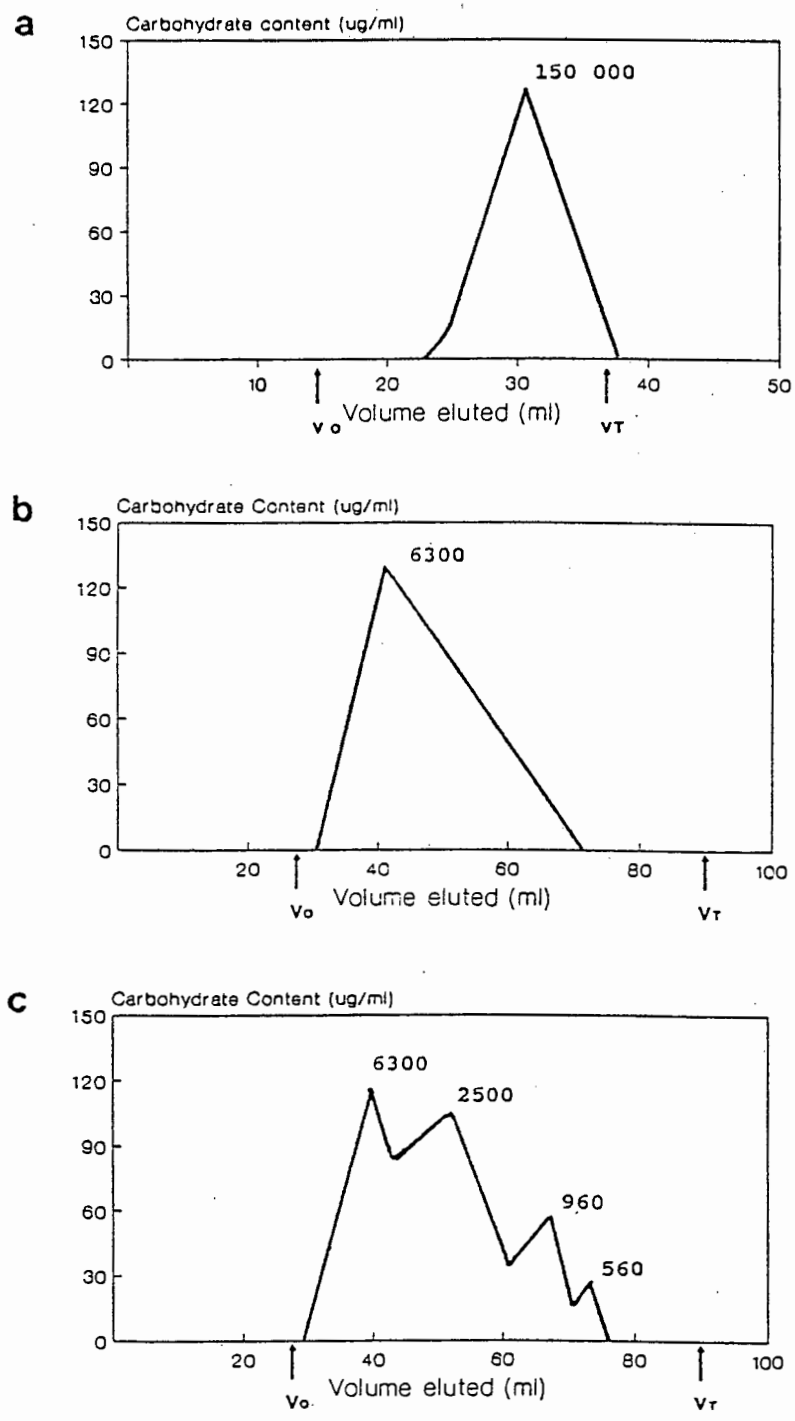



FIGURE 5. STERIC-EXCLUSION CHROMATOGRAPHY OF a. A (SEPHAROSE 4B), b. A-SD1 (BIO-GEL P-10), and c. A-SD1-METHANOL-ACETONE SOLUBLE PRODUCT (BIO-GEL P-10)

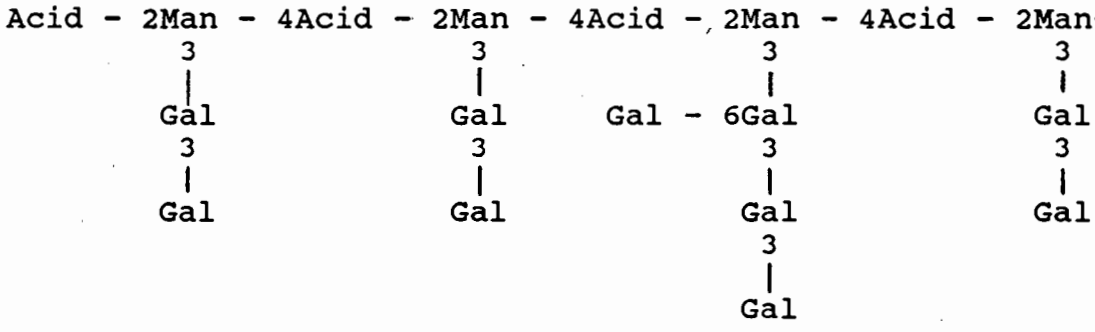
### 3.4.3 **Smith Degradation of Partially Hydrolysed HSWS (A)**

On Smith degradation of A (Scheme 5) the product A-SD1 was isolated after suitable treatment in a yield of 0.3% (Table 6). The paucity of the A-SD1 (8 mg) precluded its complete analysis but partial characterization revealed the presence of Man, Gal and GlcA in the ratio 1:1:1 with a trace of Xyl, in this product of approximately 38 sugar units, (Fig. 5.b), $\bar{M}_w$ 6300 (broad peak). The galactose was present largely as 3-linked residues with some 3,4,6-linked branch points, while the mannose was mainly 2,3-linked with a trace of terminal units.

The methanol-acetone soluble fraction (A-S) was comprised of an oligosaccharide (termed oligosaccharide-1; 0.15 g), xylose (10 mg) and glycerol (100 mg). Oligosaccharide-1, purified by chromatography on a column of Sephadex G-10 (Fig. 6.a), had $\bar{M}_w$ 4400 with a polydisperse molecular weight profile of 11000, 6000, 3700, 2000 and 960 present in mass ratio 0.4:2:1:1:0.5 (Fig. 6.b). The glycolaldehyde content (4.3%) could imply the presence of 4 periodate-resistant blocks, each with a molecular weight of 1100 on average, separated by glycolaldehyde acetal linkages. Prolonged exposure to M TFA for 56 days did not reduce the $\bar{M}_w$ as much as would have been expected when all the glycolaldehyde had been removed but the product, $\bar{M}_w$ 3200 [molecular weight distribution of 8100, 3800, 1500 and 560 present in mass ratio

ca 1:12:5:1; on Bio-Gel P-10; Fig. 6.c], comprising approximately 19 sugars, was devoid of any detectable glycolaldehyde.

Methylation analysis of this product oligosaccharide-1-56 (Table 7) showed the presence of equal proportions of end group Gal, 3-linked Gal, 2,3-linked Man and 4-linked GlcpA (each 20%) with some 3,6-linked Gal, and traces of 2-linked and 3-linked Man and end-group GlcpA (Table 7). Once again if all the glucuronic acid was linked to mannose through the 2-position,a possible representation of the structure of oligosaccharide-1 could be that shown in 7.




7

The 4-linked GlcA could have originated from 3,4-linked GlcA residues present in the partially hydrolysed product (A), to which periodate-vulnerable galactose or xylose had been attached through O-3. The formation of 7 could also result by removal of periodate-susceptible 6-linked Gal residues situated at either or both ends of this repeating unit in A. This galactose unit could be branched in HSWS, the pen-

dant Gal being removed by partial acid hydrolysis. However, no evidence has been obtained for these structural features.

TABLE 6. PROPERTIES OF SMITH DEGRADATION PRODUCTS FROM A

| PROPERTIES | A-SD1 | OLIGOSACCHARIDE-1: FIRST LIMIT | OLIGOSACCHARIDE-1: SECOND LIMIT (1-56) |
|---|---|---|---|
| $[\alpha]_D$ | + 72° | + 72° | + 78° |
| Initial weight (g) | 2.5 | 2.5 | 0.15 |
| Product weight (g) | 0.008 | 0.15 | 0.10 |
| Yield (%) | 0.32 | 6 | 4 |
| Glycolaldehyde (%) | 6 | 4.3 | - |
| Periodate consumption mmol $g^{-1}$ | 6 | - | - |
| Mol. wt. distribution | 6 300[a] (mono-disperse) | 6 100<br>3 700<br>2 000<br>960 | 3800<br>1500 |
| $\bar{M}_w$[a] | 6 300 | 4 400 | 3 200 |
| Acid equivalent | 600 | Not analysed further | 920 |
| Sugars (mol %): | | | |
| Glycerol | - | | tr |
| Erythritol | - | | tr |
| Xyl | 5 | | - |
| Man | 30 | | 28 |
| Gal | 33 | | 51 |
| GlcA[b] | 32 | | 21 |

a Determined on Bio-Gel P-10 column
b Calculated as anhydride form.

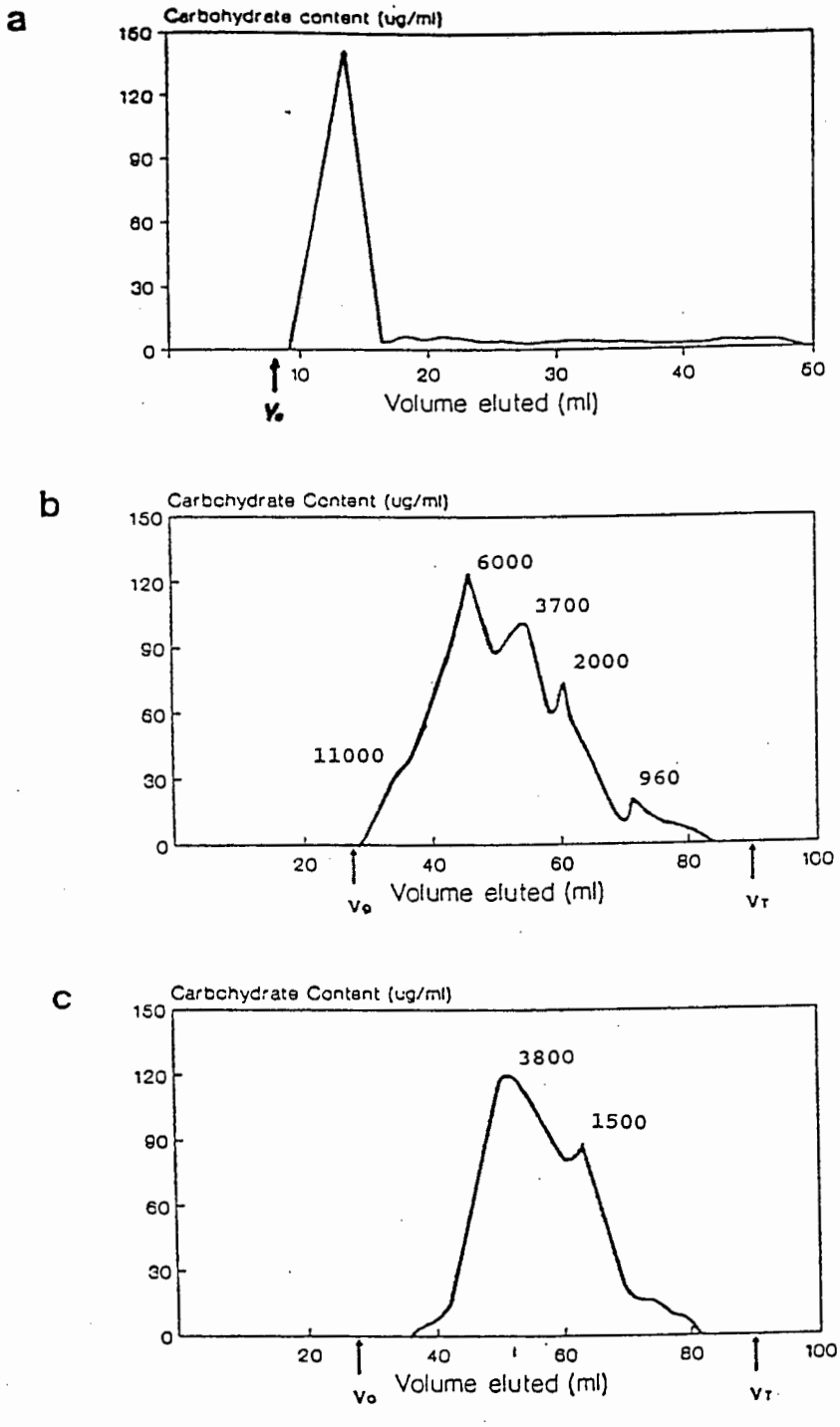



FIGURE 6. STERIC-EXCLUSION CHROMATOGRAPHY OF SMITH-DEGRADATION PRODUCTS FROM a. A-SD1 METHANOL-ACETONE SOLUBLE PRODUCT (SEPHADEX G-10), b. OLIGOSACCHARIDE-1 (BIO-GEL P-10), AND c. OLIGOSACCHARIDE-1-56 (BIO-GEL P-10)

TABLE 7. METHYLATION ANALYSIS DATA FOR A-SD1 AND OLIGOSACCHARIDE-1-56

| PARTIALLY METHYLATED SUGAR | METHYLATED: A-SD1 (mol %) | OLIGOSACCHARIDE-1-56 (mol %) |
|---|---|---|
| 2,3,4-Xyl | 4 | - |
| 2,3,4,6-Man | 1 | 1 |
| 3,4,6-Man | tr | 1 |
| 2,4,6-Man | tr | 3 |
| 4,6-Man | 16 | 20 |
| 2,3,4,6-Gal | 2 | 25 |
| 2,4,6-Gal | 30 | 20 |
| 2,3-Gal | tr | - |
| 2,4-Gal | 3 | 7 |
| 2-Gal | 10 | 2 |
| 4-Gal | tr | - |
| 2-Glc ($d_2$)[a] | n.d. | 1 |
| 2,3-Glc ($d_2$)[a] | n.d. | 19 |
| 2,3,4-Glc ($d_2$)[a] | n.d. | 1 |

a Determined after LAD reduction of methylated product

### 3.4.4 Characterisation of Hakea sericea Gum Polysaccharide Less-Soluble Fraction (HSLS)

The H.sericea less-soluble fraction, which comprised 40% of the polysaccharide gum exudate, was very similar in sugar composition (Table 1) and methylation data (Table 2) to the water-soluble (HSWS) fraction. However, it was decided to investigate this fraction in more detail since its incomplete dissolution in water at room temperature suggested possible heterogeneity of composition (although conformational differences could be another possible cause). Aspinall and McNab showed that the polysaccharide gum exudate from the North African tree Anogeissus leiocarpus, which had a glucuronomannan structure present, actually contained components (gums) of different types[92].

Since not only the HSWS fraction but also its autohydrolysed (partially hydrolysed) product were studied using the Smith degradation procedure, it was decided to repeat with the HSLS fraction the approach of Smith degradation after partial acid hydrolysis with the HSLS fraction.

The sugar composition (Table 1) of HSLS was shown to include mannose and glucuronic acid in a ratio of 1:1 (7% each), which once again indicated the possibility of the glucuronomannan type of structure in this polysaccharide. Methylation analysis before and after LAD reduction, in the same manner as

for HSWS, indicated a very similar if not identical structure for these two fractions. The GlcA was again 4-linked and 3,4-linked but the ratio (2:3) was slightly different from that of the HSWS fraction (1:3; Table 2). Smith degradation of an autohydrolysed fraction was undertaken in attempting to obtain more conclusive results as to the homogeneity of the gum exudate from H. sericea.

### 3.4.5 **Partial Acid Hydrolysis of HSLS**

Partial hydrolysis (Scheme 6) with dilute sulphuric acid gave a product A-LS (Table 4) of $M_w$ 900 000 (Fig. 7.a). Most if not all of the mannose was located in A-LS since none was found in the ethanol-soluble fraction. Paper chromatography of the latter showed the presence of arabinose (2+) with traces of xylose and galactose.

Methylation analysis of A-LS (Table 5) showed the major residues to be terminal Gal, 3-linked Gal, 6-linked Gal and 3,6-linked Gal in a ratio 4:2:5:1.

The major difference from the partially-hydrolysed HSWS was the prominence of the 3-linked Gal. However methylation analysis after LAD-reduction indicated the presence of GlcA as 4-linked and 3,4-linked residues (in ratio 2:3), and proportions of permethylated sugars differed from those found in

similar analysis of the HSWS product. This difference could be due to a lower degree of cleavage by acid of residues from the innermost core of A-LS, specifically those attached to O-2 and O-3 of GlcA, thus explaining the preponderance of 3,4-linked GlcA over 4-linked GlcA in A-LS ($\bar{M}_w$ 900 000) and the reverse of this in A ($\bar{M}_w$ 150 000).

Pendant galactose would almost certainly have been cleaved from HSWS on partial hydrolysis to the A product if the lower molecular weight of A is any guideline (although a dramatic reduction in mol.wt. is usually the result of cleavage in the chain of the inner core.)

### 3.4.6 Smith Degradation of Partially Hydrolysed HSLS (A-LS; Scheme 7)

Smith degradation gave a fraction soluble in 1:3 methanol-acetone (yield 33%) and an insoluble product which, after Sephadex G-10 column chromatography to remove salts, was isolated in a yield of 27%. The SD1 product (A-LS-SD1; Table 8) contained equimolar amounts of mannose and glucuronic acid together with 4% glycolaldehyde which could indicate the presence of periodate-resistant units held together by erythronic acid-glycolaldehyde bridges which are resistant to M TFA over the usual period of exposure of 5 days. The molecular weight profile on size exclusion chromatography

(Fig. 7.b) was polydisperse with $\bar{M}_w$ 6600 (molecular weight distribution of 11000, 7100, 5000 and 3100 present in mass ratio 1:0.5:1:1). The average proportion of glycolaldehyde was thus 4.4 moles per mol of polysaccharide.

Prolonged exposure of an aliquot of A-LS-SD1 for 50 days in M TFA yielded a product, A-LS-SD1-50 (yield 40%) with $\bar{M}_w$ 3800, of molecular weight distribution (Fig. 7.c) 6300, 4000, and 2900 in mass ratio 2:1.5:0.8, free of glycolaldehyde and with sugar composition not significantly different from that of A-LS-SD1 (Table 8).

LAD reduction of the methylated A-LS-SD1-50 product followed by hydrolysis and g.l.c-m.s. of the prepared alditol acetates revealed the presence of a trace of monomethylerythritol **8**. This can be derived from acetal-linked erythronic acid[33], as shown in Fig. 8.

TABLE 8. PROPERTIES OF SMITH-DEGRADATION PRODUCTS A-LS-SD1 AND A-LS-SD1-50

| PROPERTIES | A-LS-SD1 | A-LS-SD1-50 |
|---|---|---|
| $[\alpha]_D$ | +73° | +74° |
| Starting wt. (g) | 15 | 4 |
| Insoluble product wt. (g) | 4 | 2.4 |
| INSOLUBLE PRODUCT : | | |
| $\bar{M}_w$[a] | 6600 | 3800 |
| Acid equivalent | 750 | 700 |
| Sugar proportions (mol%): | | |
| Man | 25 | 21 |
| Gal | 50 | 54 |
| GlcA[b] | 25 | 25 |
| Glycolaldehyde[c] (%) | 4.2 | - |

a Determined on a column of Bio-Gel P-10
b. Calculated as the anhydride form
c. Determined colorimetrically by method of Dische[93]

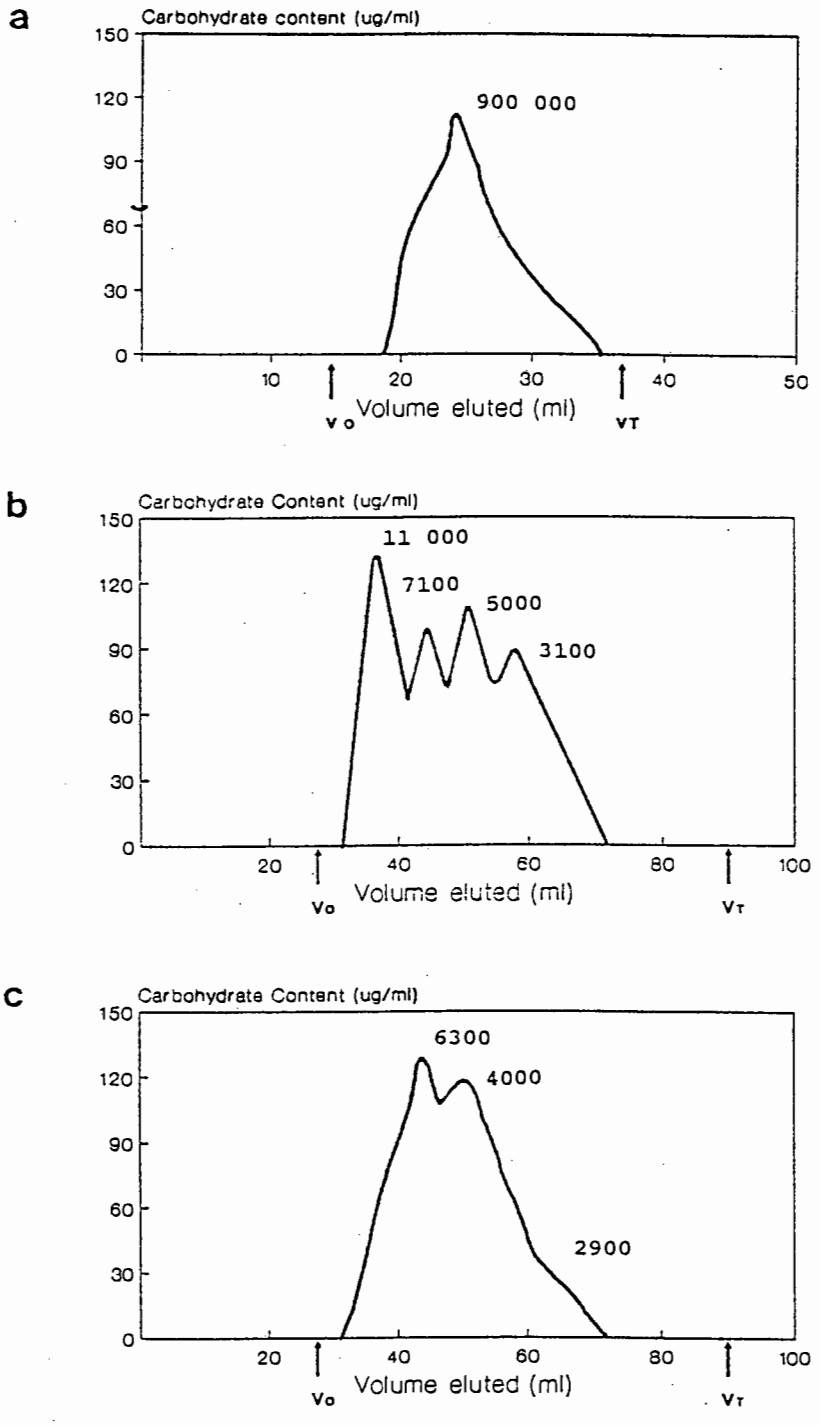
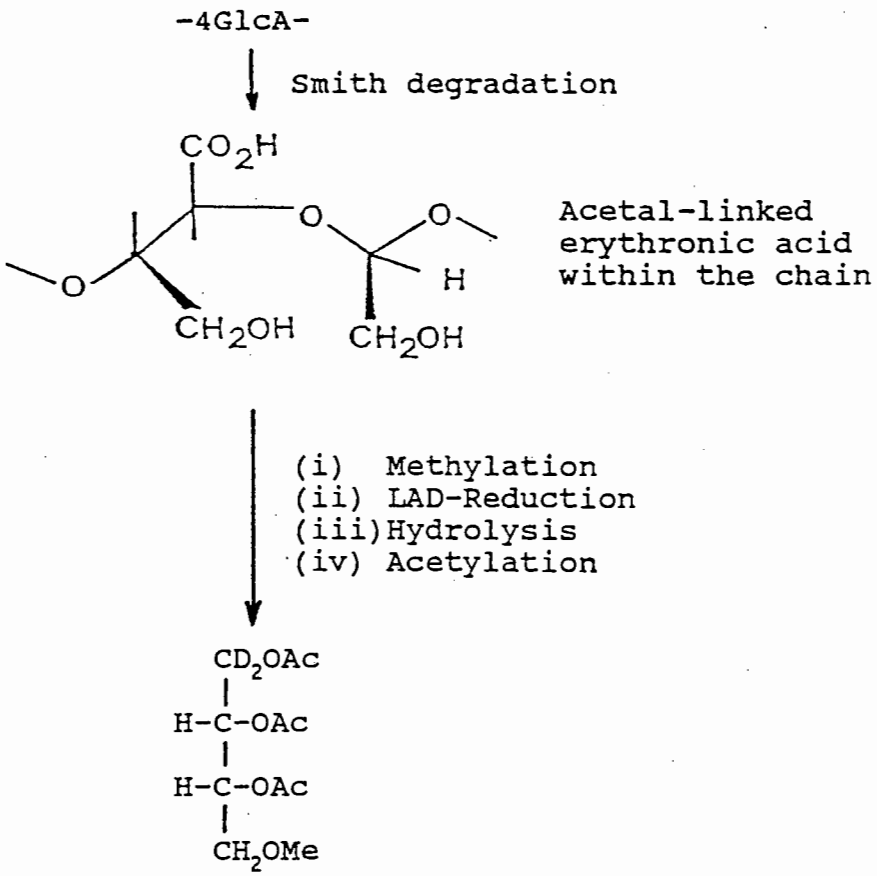


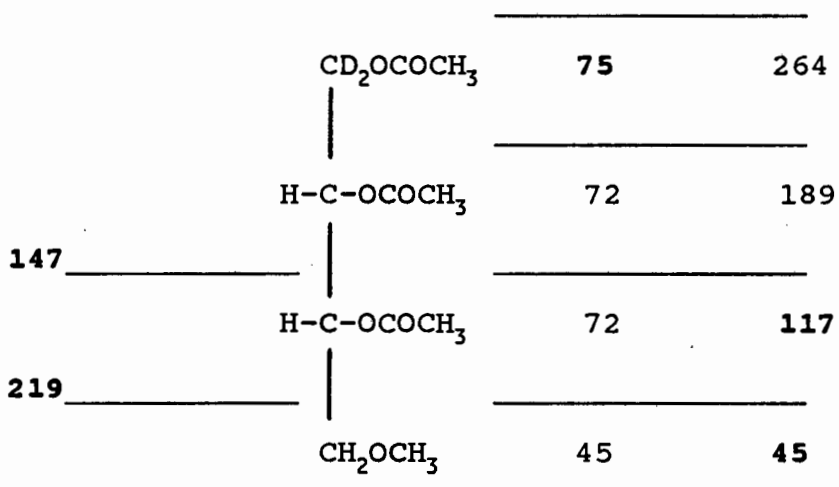

FIGURE 7. STERIC-EXCLUSION CHROMATOGRAPHY OF a. A-LS (SEPHAROSE 4B), b. A-LS-SD1 (BIO-GEL P-10), AND c. A-LS-SD1-50 (BIO-GEL P-10)




**8**

FIGURE 8. PRODUCTION OF 1-O-METHYLERYTHRITOL AS ACETATE (**8**) FROM SMITH-DEGRADED AND METHYLATED A-LS-SD1-50

The mass spectrum of the monomethylerythritol triacetate showed the characteristic ions at m/z 43(100%), 45(35), 57(5), 75(5), 87(30), 102(35), 117(75), 129(25), 144(20), 147(10), 162(5), 177(5) and 219(5). The m/z values (in bold print) of the primary fragments are indicated in **9**. The other m/z values quoted before indicate secondary fragments which are formed by the loss of unit(s) such as formaldehyde (30), methanol (32), ketene (42) and acetic acid (60).




**9**

TABLE 9. METHYLATION ANALYSIS DATA OF A-LS-SD1 AND A-LS-SD1-50

| PARTIALLY METHYLATED SUGAR | A-LS-SD1 (mol %)[a] | A-LS-SD1-50 (mol%)[a] |
|---|---|---|
| 1-O-methyerythritol | - | tr |
| 2,3,4,6-Man | 5 | 4 |
| 2,4,6-Man | 3 | 3 |
| 4,6-Man | 26 | 25 |
| 2,3,4,6-Gal | 25 | 20 |
| 2,4,6-Gal | 20 | 23 |

Also present in trace amounts in both methylated samples were 3,4,6-Man, 2,4-Gal, 2-Gal and 4(3)-Gal.
a Figures corrected for GlcA (25%)

Methylation analysis, before and after DBU base degradation of the A-LS-SD1 product (Table 9) , and base degradation using DBU and potassium-dimsyl of A-LS-SD1-50 (Table 10), indicated the following structural features:

(i) The product of Smith degradation is composed of 4 major constituent units in equal proportions (~22% each), viz. end-group Gal, 3-linked Gal, 4-linked GlcA and 2,3-linked Man with traces of end-group Man, 3-linked Man, 2-linked Man, 3,6-linked Gal, 3,4,6-linked Gal and 2,3,6-linked Gal.

(ii) Base degradation with potassium dimsyl (see Table 10 ; column 4) increased the concentration of 2,4,6-tri-O-methyl mannose (labelled at O-2 with deuterium) by 19% with a concomitant drop in 2,3-linked mannose of 23% showing that most or possibly all of the glucuronic acid degraded by the dimsyl base was linked to the O-2 position of the 2,3-linked mannose.

(iii) DBU degradation of A-LS-SD1[20,33] involved the base degradation of esterified and methylated A-LS-SD1 with identification of fragments both exterior and interior to the points of cleavage of esterified GlcA units. Sugars (mannose ethers), detached by β-elimination from positions exterior to the acidic residues, were labelled with deuterium at C-1 by borodeuteride reduction before the removal, by mild acid hydrolysis, of the unsaturated acidic moieties remaining attached to interior sugar units. In this sequence the alditol (mannitol) formed by borodeuteride reduction was subsequently further labelled by deuteriomethylation at positions 1 and 5 of the ring-opened exterior sugar unit. If the exterior contiguous residue was acid, the position to which this acid was attached, i.e. the O-2 position of mannose, was also deuteriomethylated, whereas if a neutral sugar was contiguous the position of attachment was eventually acetylated after complete hydrolysis and derivatization to alditol acetates.

TABLE 10. RESULTS OF BASE-DEGRADATION OF A-LS-SD1 AND A-LS-SD1-50

| PARTIALLY METHYLATED SUGAR (AS ALDITOL) ACETATES) | SAMPLES[a,b] 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | (mol %) | (mol %) | (mol %) | (mol %) |
| Derivatised mannitol[c] (Structure 10) | - | 1 | 10 | - |
| 2,3,4,6-Man | 5 | 6 | 9.5 | 6 |
| 2,3,4,6-Gal | 25 | 4.3 | 16.5 | 20 |
| 2,4,6-Man | 3 | 6 | 14 | 22 |
| 2,4,6-Gal | 20 | 14 | 17 | 28 |
| 4,6-Man | 26 | 9 | 12 | 3 |
| 2,3-Gal | - | - | - | tr |

2,4-Gal, 2-Gal and 4(3)-Gal were present in trace amounts in all samples.

a Samples of products of Smith Degradation viz:
1. Methylated A-LS-SD1
2. DBU-degraded A-LS-SD1
3. DBU-degraded A-LS-SD1-50
4. Dimsyl-degraded A-LS-SD1-50

b Figures quoted have all been corrected for GlcA 21% (anhydride form)

c Structure 10 as in text

These changes are as follows

-4)- Sugar-(1-2)- Man -(1-4)- GlcA -(1-

(i) DBU-catalysed degradation

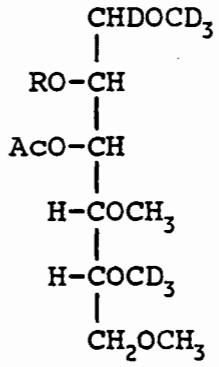
(ii) borodeuteride reduction

(iii) hydrolysis of unsaturated acid residues

(iv) deuteriomethylation

(v) hydrolysis

(vi) borohydride treatment and acetylation

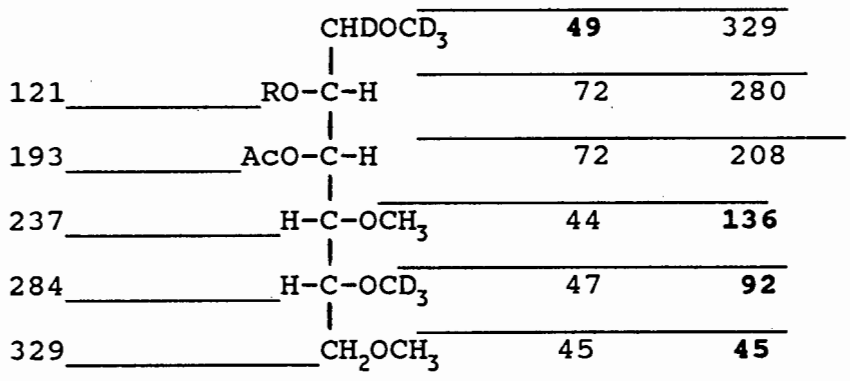



**10**

R = Ac where sugar is exterior to the Man as shown
R = $CD_3$ where GlcA is exterior to the Man residue.

The derivatised mannitol corresponding to **10** that was identified by g.l.c.-m.s. in the products of DBU degradation of methylated A-LS-SD1 showed definitive ions in the mass spectrum (% in parentheses):

m/z 43(100%), 45(20), 49(60), 81(30), 82(20), 84(50), 85(55), 92(12), 95(30), 97(55), 99(30), 101(15), 104(60), 109(20), 125(25), 127(15), 136(12), 139(15) and 269(15); m/z values of the primary fragments (in bold print) are shown in **11**. These results indicate acetylation occurred at position 2.




**11**

R = $COCH_3$ (m/z 72); [or $OCH_3$ (m/z 44)-however no evidence was found for its presence].

The results of g.l.c.-m.s. analysis of the derivatised products from A-LS-SD1 (Table 10) show the juxtaposition of mannopyranosyl (M) with respect to contiguous acid (GlcA) or sugar (S) residues to vary as follows:

(a) Complete disappearance of the small amount of 2-linked Manp and a 1% increase in the molar proportion of terminal Manp with the introduction of $-OCD_3$ groups at position 2 reflect the presence of the sequence GlcA-2M-S. However S in this structure would appear as present if any undegraded GlcA exists in the oligosaccharide. Thus the result here could be ambiguous as not all the GlcA was degraded during this procedure.

(b) The molar proportion of 2,3-linked Manp fell from 26 to 9% whilst:

(i) the proportion of 3-linked Manp rose from 3 to 6%, with the introduction of $-OCD_3$ groups at position 2, corresponding to the sequence

GlcA-2M-S
3

Similarly here ambiguity exists about S being genuine or actually a GlcA which had escaped degradation;

(ii) there was a new mannitol component in a concentration of 1 mol % with structure **10**, which indicated the occurrence

of the sequence

S-2M-GlcA
3

Qualitatively these results indicate the occurrence of GlcA-2M-S and the S-2M-GlcA sequences, but there is no evidence for the GlcA-2M-GlcA sequence found in Grevillea robusta gum[33]. Similar conclusions can be drawn from the DBU treatment of the A-LS-SD1-50 product (Table 10; column 3). In both cases there are indications of structural significance but not quantitative accuracy, since not all the acid was degraded. However, the decrease in 2,3-linked mannose after dimsyl treatment was more complete than that caused by DBU degradation, indicating the more effective degradative action of the former on GlcA under the conditions used in the present work. Possibly longer exposure to DBU than the 16 h used here is required[32].

### 3.4.7 Summary of Results of Smith Degadation of H.sericea Gum

From these experimental results it may be concluded that the HSWS and HSLS fractions of the H. sericea gum exudate were not significantly different, the major variations being those shown by the methylation analyses, viz the large proportion of 3-linked Gal and the larger ratio of 4- to 3,4-linked GlcA in HSLS. The two fractions were similar in

sugar composition, specific rotation, and behaviour on autohydrolysis, methylation and Smith degradation. Thus the gum from H. sericea appears to be homogeneous with respect to composition and molecular structure, in contrast to gum ghatti[19].

The different water solubilities of these two fractions may be the result of different degrees of hydration in the different layers of the gum nodules. While on the bark of the tree, the nodules are exposed to a wide range of atmospheric conditions which could cause hardening of the outer layers to a greater degree than the innermost ones. These layers then dissolve at very different rates during the cold water-dissolution stage of polysaccharide isolation, but the difference is significantly lessened when the water is heated.

## 3.5 PREPARATION AND CHARACTERIZATION OF 2-O-( β -D-GLUCOPYRANOSYLURONIC ACID)-D-MANNOSE FROM HSLS

A preliminary partial hydrolysis experiment was performed to confirm the presence of and to isolate the aldobiouronic acid GlcA-2Man from HSLS, as in a previous study of

*H. acicularis* gum[45]. This isolation was achieved, albeit in low yield (2%), by treatment of HSLS with 0.05 M sulphuric acid for 20 h at 100°C, followed by neutralisation, centrifugation and freeze-drying of the supernatant solution.

TABLE 11. PROPERTIES OF THE ALDOBIOURONIC ACID, 2-O-( β -D-GLUCOPYRANOSYLURONIC ACID)-D-MANNOSE FROM HSLS

| DETERMINATION | | VALUE | |
|---|---|---|---|
| i. | Mol.wt. | 360[a] | |
| ii. | $[\alpha]_D$ | -34° (c 1.3) | |
| iii. | $R_{Gal}$ (solvent C) | 0.60 | |
| iv. | GlcA (%) | 50[b] | |
| v. | **Methylation data:** | | |
| | $[\alpha]_D$ | -36.6° (c 2.1) | |
| | methyl ethers | 3,4,6-Man[c] | |
| vi. | **LAD-reduced methylated GlcA-2Man data :** | | |
| | $[\alpha]_D$ | -20° (c 1.1) | |
| | methyl ethers[c] | 3,4,6-Man | 50 |
| | (mol %) | 2,3,4-Glc($d_2$) | 50 |

a Determined on a column of Bio-Gel P-2
b Determined by the method of Blumenkrantz[85]
c Determined by g.l.c. and g.l.c.-m.s. on column B

Preparative chromatography using Sephadex G-10 followed by Bio-Gel P-2 afforded this aldobiouronic acid in a purified form which facilitated characterisation (Table 11).

N.m.r. experiments utilising both 1-D and 2-D methods showed anomeric proton signals at $\delta$ 5.27 (0.75H, $J_{1,2}$ = 1.4 Hz) and 4.96 (0.25H, singlet) indicating the presence of $\alpha$- and $\beta$ -Manp. Also, the anomeric signals at $\delta$ 4.56 (0.75 H, $J_{1,2}$ = 7.6 Hz) and $\delta$ 4.67 (0.25 H, $J_{1,2}$ = 8.4 Hz) indicated the presence of $\beta$ -GlcpA in the $\alpha$ - and $\beta$ -anomers of the disaccharide respectively[94].

## 3.6 PREPARATIVE PARTIAL HYDROLYSIS OF *Hakea sericea* WHOLE GUM (Scheme 8)

The relationship between GlcA and Man in the core of the polysaccharide has been shown by sequential Smith degradation and hydrolysis to be such that they form at the least single units of the aldobiouronic acid, 2-*O*-( $\beta$ -D-glucopyranosyluronic acid)-D-mannose. The possible occurrence of multiple aldobiouronic acid units, either interspersed with other sugars in the polysaccharide structure or linked contiguously in a chain, as found in the glucuronomannoglycan type of polysaccharide structure, was investigated by preparative partial hydrolysis.

Preliminary partial hydrolysis of an aliquot of HSWS, followed by p.c. monitoring (solvents A,B and C), showed that the largest number of acidic oligosaccharides were released after 10 h of exposure to sulphuric acid (0.5 M; 96°C). The bulk sample (50 g) was similarly hydrolysed (Scheme 8). After neutralisation ethanol was added to the hydrolysate solution to yield two products, one ethanol-soluble (yield 76% by wt.) and the other insoluble (10% by wt.) in ethanol. Examination of the soluble product (syrup) by p.c. (solvent C) revealed the presence of the following components in proportions indicated as approximate percentages (by wt.) of the total carbohydrate in the polysaccharide: two oligosaccharides with $R_{Gal}$ values equal to 0.51 (14; mobility equal to Gal-6Gal) and $R_{Gal}$ 0.61 (7; mobility equal to GlcA-2Man), Gal (40), Ara (19) and Xyl (7); free mannose was absent. However, the yields indicated that destruction of a minor proportion of GlcA and Man must have occurred. The monosaccharides in the soluble product were probably located in the periphery of the structure as indicated by the autohydrolysis experiments already described (Section 3.4.5).

The bulk of the insoluble product (4.6 g) was separated by cellulose column chromatography into 80 fractions (Table 12). However, none of these fractions contained only one component. Fractions 4 to 20 (3.91 g), containing GlcA-2Man in addition to slower-moving oligosaccharides, were pooled (fraction PF) and again subjected to cellulose column

chromatography. Paper chromatography (solvent C) of fraction PF revealed the presence of components with mobilities very similar to those present in fractions 1 to 3 (0.036 g) and fractions 21 to 80 (1.488 g), and also very similar to some of the acidic components present in the acid hydrolysate of E. longifolius gum (Table 13)[30,31], notably GlcA-2Man and its dimer and trimer. The pooled fraction PF was separated by graded elution (Fig. 10) into 240 sub-fractions. After examination by p.c. and in certain cases additional tests performed at a later stage, these were pooled into a total of 17 fractions. The fractions were quantified (% of total sample in parentheses), characterised and found to be composed of the following : Xyl (0.02), GlcA (1.31), Gal (0.386), Man (0.016), 2-O-( β -D-glucopyranosyluronic acid)-D-mannose (monomer; **3**; 4.42), GlcA-2Man3-Gal (**12**; 0.08), GlcA-6Gal-4GlcA-2Man (**13**) or GlcA-2Man-4GlcA-6Gal (**13.a**; 0.238), pure dimer GlcA-2Man-4GlcA-2Man (**14**; 0.310), dimer with trace of constituent Gal (0.426; in 2 fractions),

GlcA-2Man-4GlcA-2Man-6Gal-4GlcA-2Man (**15**; 0.088),

GlcA-2Man-4GlcA-2Man-GlcA-2Man
3
|
Gal6-Gal (**16**; 0.482),

and a crystalline product at origin (0.038).

On p.c. in a two acid-solvent system (solvent D) the monomer and dimer (and the trimer of Ornithogalum thyrsoides-see Section 3.7.6.1) had a linear relationship when the function $\log[(1/R_{Gal})-1]$ was plotted against the suspected degree of polymerisation[95].

Oligosaccharides (ca. 7.32% of the total gum) isolated during this study included those mentioned before, which comprised 5.94%, and fractions 1 to 3 and 21 to 80, which comprised the other 1.38% of the oligosaccharide content. The latter oligosaccharides were not isolated but examined on p.c. (solvent C) against the characterised oligosaccharides as authentic samples. They were all shown to be composed of compound **3** (+), dimer **14**, with a trace of Gal impurity (+), monomer (tr) and Gal (+). Chromatography on a column of the anion-exchanger Bio-Rad AG 1-X2 (formate) was no more successful in effecting separation of the acidic oligosaccharides.

Isolation of sequences longer than those obtained in this study should be possible by variation of conditions of hydrolysis, and possibly by the judicious use of enzymes such as the galactosidases. The latter enzymes could also be useful in future examination of oligosaccharides where, as with some in this study, the galactose moieties remain tightly bound to the glucuronomannoglycan core in spite of the rather harsh acidic conditions used to generate them. These galac-

tose residues are not easily removed by further partial hydrolysis to yield the remaining part of the oligosaccharide as an intact unit.

The presence of the trimer in structure **16** hints strongly that at least half if not all, of the GlcA and Man residues are situated contiguously and alternately in the core of the Hakea sericea polysaccharide structure.

## 3.7 INVESTIGATION OF THE POLYSACCHARIDE EXUDATE FROM *Hakea sericea*. (ii) EXPERIMENTAL

### 3.7.1 Methylation Analysis

The polysaccharide was methylated as described in Section 2.7. It was deionised and methylated by the method of Hakomori[67] and by three treatments with Purdie reagents. The p.m.a.a.s were analysed by g.l.c. and g.l.c.-m.s., and quantitation was achieved by the use of the response factors of Sweet *et al*[81].

### 3.7.2 Smith Degradation of HSWS

The general experimental procedure used in this study was as follows :

The vacuum-dried (40°C) polysaccharide sample (1% solution in 0.12 M $NaIO_4$) was allowed to stand in the dark at room temperature. Aliquots (1.0 mL) were removed at intervals (not less than once in 24 h) and assayed titrimetrically with standard arsenite[65] to determine periodate consumption. Sampling and titration were continued until the concentration of periodate had remained constant for 48 h. The excess periodate ions were removed from solution by either of two methods:

(i) The addition of an excess of ethan-1,2-diol, which was oxidised to formaldehyde, the periodate being reduced to iodate. These products were removed by dialysis against distilled water, which was replaced daily until dialysis was completed. Each dialyzate was tested for the presence of low molecular weight carbohydrate by analysis of a concentrated aliquot viz. 20 % of the dialyzate concentrated to a small volume (50 mL). The iodate was removed by addition of barium acetate followed by centrifugation to remove the precipitated barium iodate, and other ions by deionization with Amberlite IR-120 ($H^+$) ion- exchange resin. After concentration of the dialysable fractions to 50 mL, aliquots were tested for carbohydrate by the Molisch test (qualitatively) and the phenol-sulphuric acid method (quantitatively), so as not to lose any significant amount of carbohydrate product. Acidified KI solution was used to test qualitatively for iodate.

(ii) The addition of barium acetate to remove the iodate and periodate ions as precipitates which were collected by centrifugation. This method was used in cases where the low $\bar{M}_w$ of the degraded products precluded dialysis. The precipitate was washed thoroughly with distilled water to retrieve any adsorbed carbohydrate, and the supernatant and washings were pooled.

The next phase of the Smith degradation was the reduction process where sodium borohydride was added to the solution of oxidized polymer, adjusted to pH 7 with sodium bicarbonate. The iodate produced during reduction was monitored as detailed above. After reduction was completed, acetic acid was added slowly to decompose the borohydride (pH 4). Freeze-drying was followed by removal of borate ions in the form of volatile trimethylborate, achieved by successive evaporations with methanol containing 1% acetic acid. The residue was monitored for borate by the flame test using concentrated $H_2SO_4$ and methanol.

The reduced-oxidised product was then subjected to mild hydrolysis at room temperature with M TFA, to break labile acetal linkages. The hydrolysis was monitored by subjecting aliquots (1 mL), neutralised with $NaHCO_3$, to steric-exclusion chromatography on a column packed with material of appropriate fractionation range. These aliquots were tested at 24 h intervals, until $\bar{M}_w$ of the carbohydrate was shown to be constant for 48 h. The solution was then freeze-dried and the product fractionated by the addition of a methanol-acetone mixture in proportions ranging from 1:1 to 1:3 v/v, depending on the molecular-weight distribution of the products. This usually gave an insoluble fraction which was recovered by centrifugation, dissolved in water and freeze-dried. Residual salts were then removed from this

product by dialysis, but for products of low $\bar{M}_w$ chromatography on a column packed with suitable material such as Sephadex G-10, with water as eluent, was used. After purification in this way, the product insoluble in the methanol-acetone mixture was obtained by dissolving in water and freeze-drying. The fraction soluble in this mixture was treated with Amberlite IR-120 ($H^+$) resin, filtered and evaporated to a syrup or freeze-dried. These SD products were then analysed by various methods as described below.

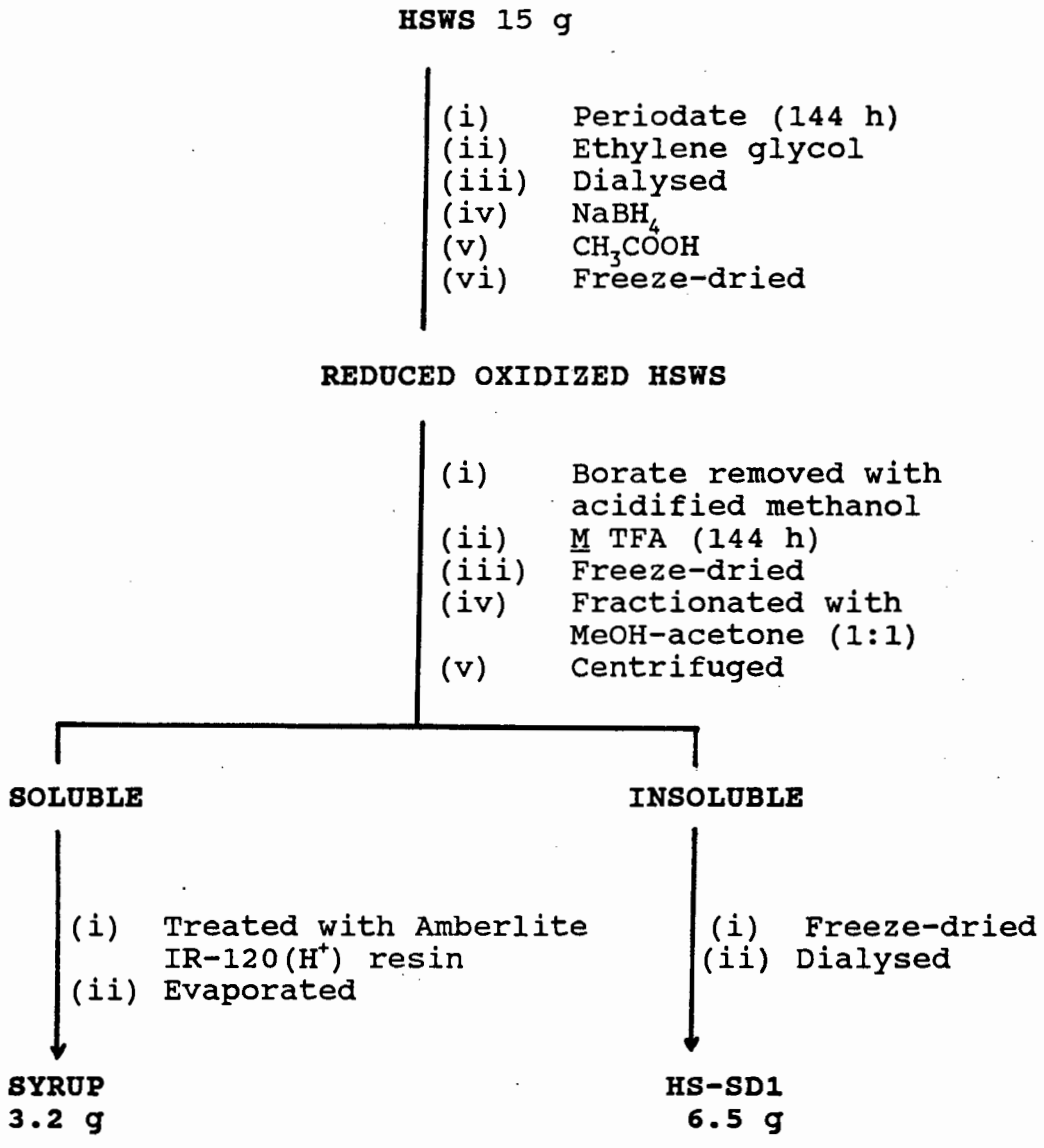
### 3.7.2.1 **First Smith degradation of HSWS (Scheme 1)**

Oxidation of HSWS (15 g) for 144 h, resulted in a periodate consumption of 6.0 mmol $g^{-1}$ which was not significantly different from the calculated value of 6.4 mmol $g^{-1}$. Treatment to remove excess periodate was followed by dialysis against distilled water (5 L) which was changed at intervals and monitored for carbohydrate as described. These tests were negative for all dialysates. After further dialysis the retentate solution was subjected to reduction with $NaBH_4$ for 144 h after which time the borate was removed in the usual manner to give the reduced oxidized product.

Hydrolysis with M TFA was allowed to proceed for 144 h, the molecular-weight distribution of the carbohydrate products being constant (Fig. 3.b) after 96 h. Fractionation by

addition of 1:1 (v/v) methanol-acetone yielded two products, viz. the insoluble SD1 product (HS-SD1; 6.5 g) and the syrupy soluble fraction (3.2 g).

SCHEME 1 FIRST SMITH DEGRADATION OF HSWS



Fraction HS-SD1 was characterised by chromatography on Sepharose 4B (Fig. 3.b), acid hydrolysis and g.l.c. to determine the sugar composition, methylation analysis, and further Smith degradations. The fraction soluble in 1:1 (v/v) methanol-acetone was examined by p.c. (solvents A, B and C) using both p-anisidine hydrochloride and alkaline Ag $NO_3$ as detection reagents. This showed the presence of glycerol (5+) and arabinose (+) with xylose (tr).

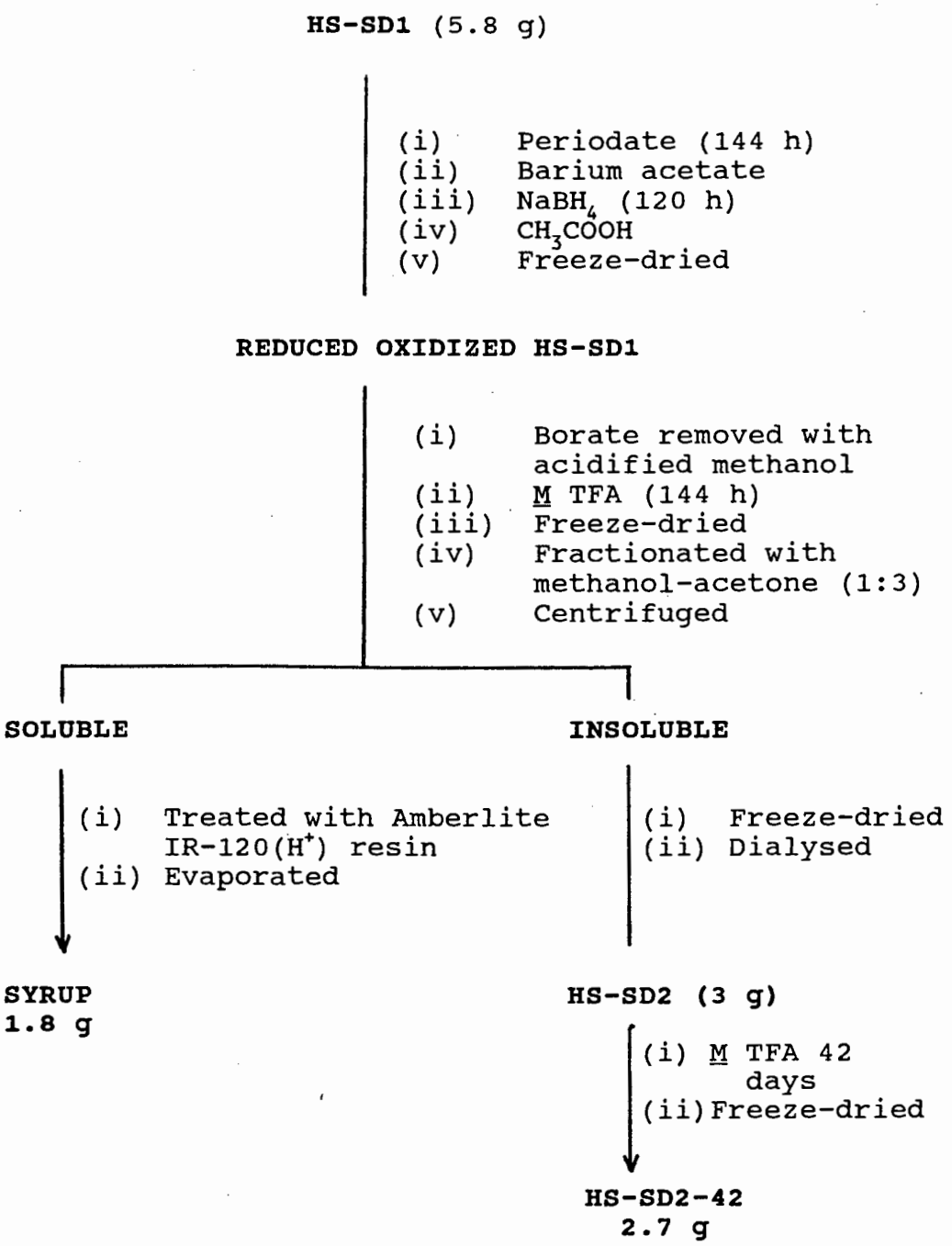
### 3.7.2.2. **Second Smith degradation (Scheme 2)**

On oxidation of HS-SD1 (5.8 g) in the usual manner, the periodate consumption became constant (6.0 mmol $g^{-1}$) after 144 h. This figure was not significantly different from the theoretical value of 5.5 mmol $g^{-1}$.

The excess periodate and iodate ions were removed by the addition of barium acetate, and reduction with $NaBH_4$ for 120 h was followed by work-up as previously described. Mild hydrolysis of acetals with M TFA, monitored by s.e.c. on a column of Bio-Gel P-10 at 24 h intervals, yielded a product with a constant $\bar{M}_w$ after 144 h. Freeze-drying and fractionation by addition of 1:3 (v/v) methanol-acetone gave an insoluble product(HS-SD2) and a soluble product which were separated by centrifugation.

The HS-SD2 product (3.0 g; Table 3) contained glycolaldehyde (*ca*. 1%) which has been shown to be part of a link between periodate-resistant blocks and thus an indication of incomplete Smith degradation. Prolonged exposure (42 days) of an aliquot to M TFA removed this glycolaldehyde yielding a product, HS-SD2-42 of lower $\bar{M}_w$ (Fig. 4.a), which was further examined by methylation analysis, before and after LAD reduction and base degradation with potassium dimsyl. The methanol-acetone soluble product after treatment with Amberlite IR-120($H^+$) was evaporated to a syrup and examined by p.c. (solvents A, B and C) which revealed the presence of glycerol (10+), Ara (+), Xyl (+) and erythritol (tr).

SCHEME 2 SECOND SMITH DEGRADATION OF HSWS.

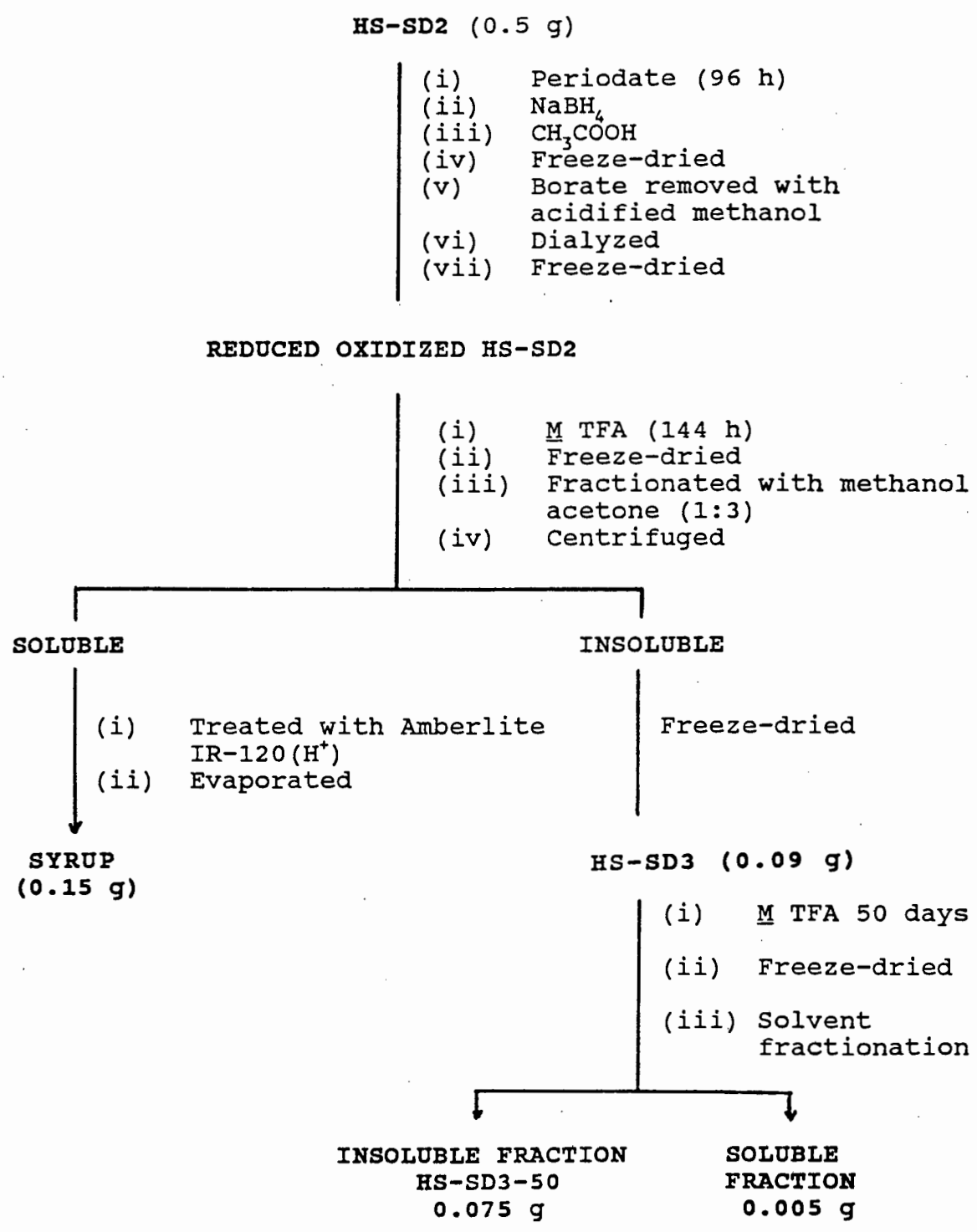


### 3.7.2.3 **Third Smith degradation (Scheme 3)**

On periodate oxidation of HS-SD2 (0.5 g) in the usual manner for 72 h, periodate consumption reached a constant level of 4.5 mmol $g^{-1}$ (18% higher than the theoretically calculated value). After 96 h, without removal of the periodate, a quantity of $NaBH_4$ (2 g) was added to reduce both oxidized oligosaccharide and excess periodate. This was done with the aim of improving the yield of the HS-SD3 product[96].

Reduction for 120 h was followed by borate removal and dialysis against distilled water using a dialysis membrane of nominal molecular-weight cut-off 3500, to remove unwanted salts. The dialyzate was tested for carbohydrate, with negative results. After treatment of the reduced-oxidised product with M TFA at room temperature for 144 h, the molecular weight remained constant, according to s.e.c. on a column of Bio-Gel P-2 (Fig. 4.b). The freeze-dried product was fractionated, by the addition of a 1:3 (v/v) mixture of methanol-acetone, into an insoluble (HS-SD3) and a soluble fraction. The HS-SD3 (90 mg) (Table 3), was subjected to further, prolonged mild hydrolysis to remove all the bound glycolaldehyde present (4.2%), so that the true molecular weight of the block of coherent, intact sugar residues could be obtained.

SCHEME 3 THIRD SMITH DEGRADATION OF HSWS



After 50 days the freeze-dried product was subjected to fractionation with a mixture of methanol-acetone (1:3 v/v) which yielded 2 products, one insoluble (HS-SD3-50; 75 mg) and the other soluble (5 mg) which was shown by p.c. to contain only galactose.

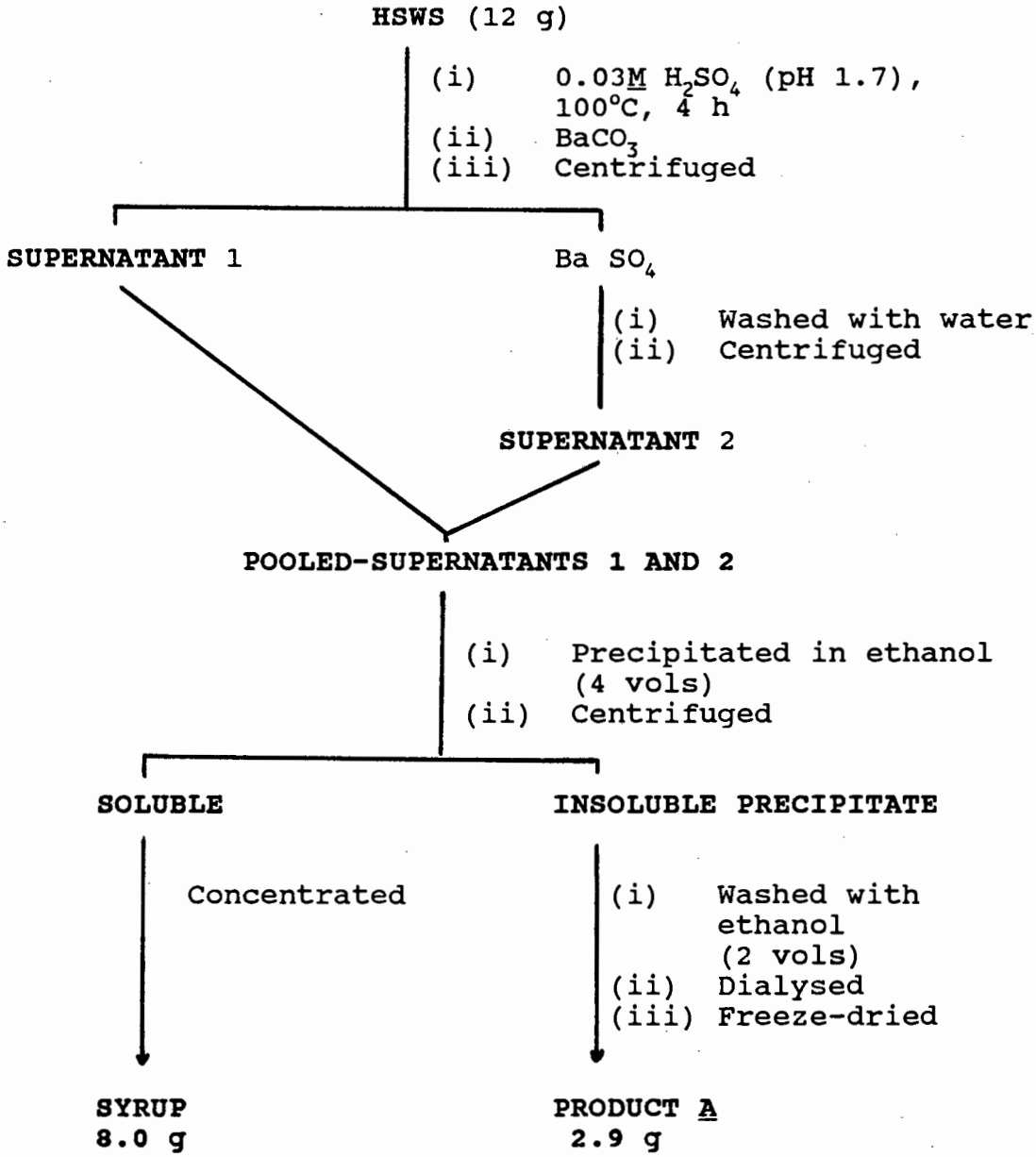
The HS-SD3-50 with $\bar{M}_w$ 1500 (Fig. 4.c), was composed of Man and Gal in a ratio 2.5 : 1 and GlcA 8%. Methylation analysis revealed the only periodate-oxidizable residues present in the HS-SD3 to be terminal Gal and a trace of terminal Man, with the GlcA being 4-linked; consequently, another Smith degradation was not indicated.

### 3.7.3 **Partial Acid Hydrolysis (Autohydrolysis) of HSWS (Scheme 4)**

A vacuum-dried (40°C) sample of Hakea sericea water-soluble fraction (HSWS; 12 g) was dissolved in 0.03 M $H_2SO_4$ (600 mL, giving a 2% solution of pH 1.7) and heated at 100°C, the hydrolysis being monitored by the change in optical rotation with time (Fig. 9). At 4 h, when there was a definite decrease in optical rotation, hydrolysis was stopped, and the solution was cooled and neutralised with $BaCO_3$. Barium sulphate was removed by centrifugation and the residue was washed with distilled water and centrifuged; the supernatants were pooled and concentrated, under reduced pressure at 40°,

to 100 mL. This solution was poured into ethanol (4 vols) with stirring, and the precipitated product was isolated by centrifugation, and washed with ethanol. All the supernatants were pooled, concentrated and retained for further examination (ethanol-soluble product).

SCHEME 4 PARTIAL HYDROLYSIS (AUTOHYDROLYSIS) OF HSWS



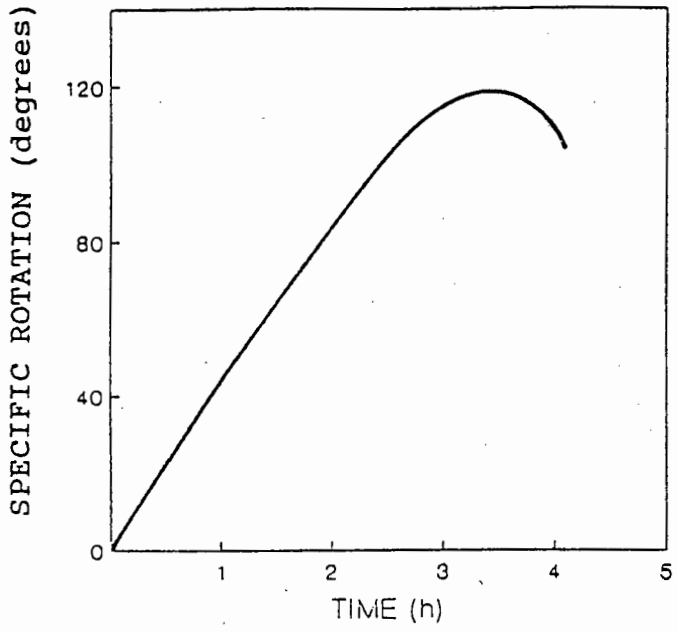



FIGURE 9. MONITORING OF PARTIAL HYDROLYSIS OF HSWS

(i) **Ethanol-insoluble fraction**

This fraction was dissolved in distilled water (200 mL) and dialyzed against distilled water (10 L) for 96 h, and the retentate was freeze-dried to give the autohydrolysed fraction, A (2.9 g; $\bar{M}_w$ 150 000, Fig. 5.a). An aliquot (1 L) of the dialyzate was concentrated to 10 mL, and tested for the presence of carbohydrate by the Molisch and phenol-sulphuric methods which proved negative. The fraction A was analysed for neutral sugars and found to be devoid of the Ara present in the whole gum (Table 4).

Methylation analysis revealed that 80% of A was composed of T-Galp, -6)-Galp, -2,3)-Manp and -4)-GlcpA in the ratio approximately 1:1:1:1 ( Table 5).

(ii) **Ethanol-soluble fraction**

An aliquot (0.5 g) of the ethanol-soluble product was dissolved in water (10 mL) and subjected to p.c. (solvent C) which revealed the presence of several components, having $R_{Gal}$ values of 0.08 (+), 0.18 (+) (same mobility as raffinose), 0.72 (+++) and 1.15 (6+) (i.e. mobility equal to that of arabinose). No acidic components were noted (solvent B).

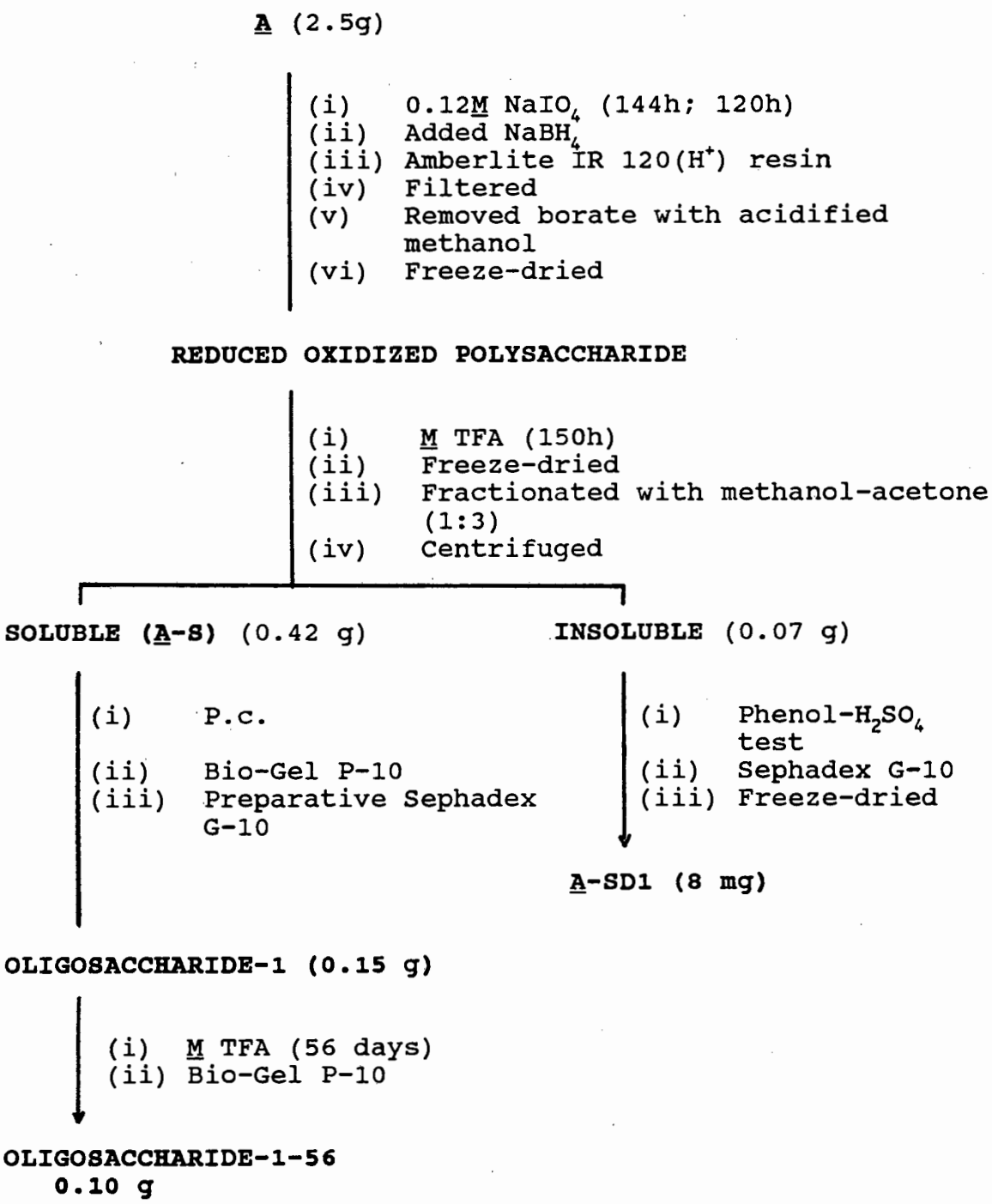
3.7.3.1 **Smith degradation of A (partially hydrolysed HSWS) (Scheme 5)**

On oxidation of A (2.5 g; Fig. 5.a) in the usual manner for 144h the periodate consumption became constant at 6 mmol $g^{-1}$ which approximately equalled the calculated value of 5.7 mmol $g^{-1}$ (Table 6). $NaBH_4$ was added and reduction of oxidised A allowed to proceed for 150 h, when the solution was decationised with Amberlite IR-120 ($H^+$) resin.

The resin was removed by filtration and the filtrate evaporated to dryness. After borate removal, freeze-drying and treatment with M TFA a constant molecular weight was reached after 150 h in M TFA (monitoring on Bio-Gel P-10).

The freeze-dried product was then fractionated by the addition of methanol-acetone (1:3 v/v) into two products, the soluble (A-S; Fig. 5.c) and insoluble (A-SD1; Fig. 5.b). The latter product, analysed by the phenol-sulphuric method, was shown to contain 12% (8 mg) carbohydrate. The salts were removed from this product by passage through a column of Sephadex G-10 using water as the eluent. The purified A-SD1 solution was freeze-dried and subjected to analysis of $\bar{M}_w$ and sugar composition, and to methylation analysis.

SCHEME 5 SMITH DEGRADATION OF PARTIALLY HYDROLYSED *H. sericea* WATER-SOLUBLE FRACTION (A)



P.c. (solvent A) of the soluble fraction (A-S; 0.42 g) revealed the presence of glycerol and an oligosaccharide located at the origin of the paper chromatogram. An aqueous solution of A-S was subjected to s.e.c. on a preparative column of Sephadex G-10 (25 X 1 cm; Fig. 6.a). The bulk of the oligosaccharide eluted as a sharp single peak and was easily separated from the glycerol-containing portion (0.270 g).

The oligosaccharide solution was freeze-dried to yield a product (oligosaccharide-1; 0.150 g) having $[\alpha]_D$ + 72°, a glycolaldehyde content of 4.3% and a wide molecular weight distribution with a weight-average molecular weight of 4400 (Fig. 6.b).

The glycolaldehyde was removed after prolonged exposure to M TFA (56 days) and the oligosaccharide purified by passage through a column of Bio-Gel P-10 (30 X 1 cm) to yield a product, oligosaccharide-1-56 (80% yield) having $\bar{M}_w$ 3200 (Fig. 6.c). Methylation analyses including reduction with LAD were performed and the results permitted postulation of structure **7**

### 3.7.4 **Partial Acid Hydrolysis (Autohydrolysis) of HSLS (Scheme 6)**

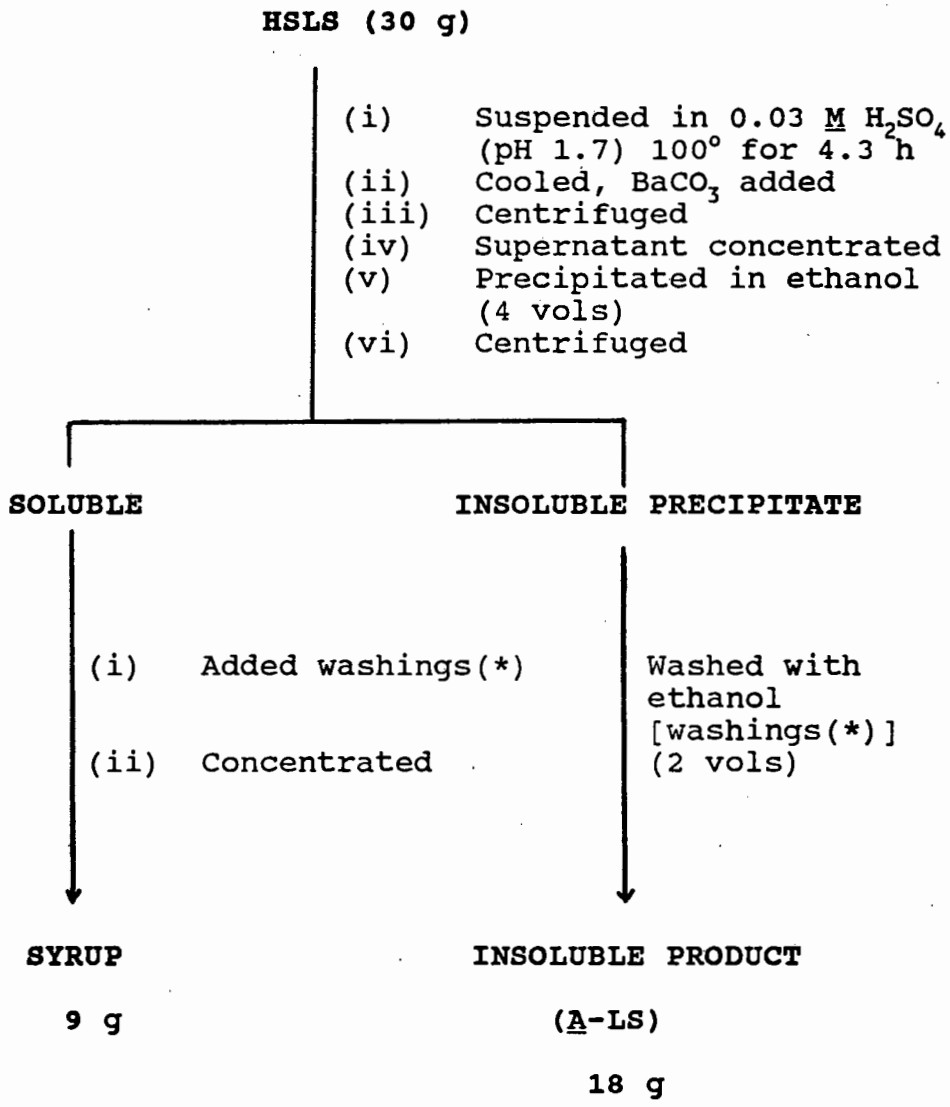
The vacuum-dried (40°C) sample of Hakea sericea less-soluble gum (HSLS; 30 g; Table 1) was suspended in 0.03 M $H_2SO_4$ (pH 1.7 ; 1.5 L) and heated at 100°C.

Hydrolysis of the dissolved polysaccharide was monitored by change in optical rotation with time. At 4.3 h hydrolysis was terminated when the specific optical rotation decreased rapidly from +90 to +80°.

The solution was cooled and neutralised with $BaCO_3$. Barium sulphate was removed by centrifugation, washed with distilled water and centrifuged. The supernatants were pooled and concentrated to 100 mL. This solution was poured into ethanol (4 vols) with stirring,and the precipitated product (A-LS) was isolated by centrifugation, and washed with ethanol, dissolved in water and freeze-dried.

All the ethanol supernatants were pooled and concentrated to yield a syrup (9 g) which was found on examination by p.c. (solvent C) to contain components with the following $R_{Gal}$ values: 0.08 (+), 0.18 (+), 0.43 (+), 0.78 (++) and 1.10 (6+ ; mobility equal to that of arabinose).

SCHEME 6 PARTIAL HYDROLYSIS (AUTOHYDROLYSIS) OF HSLS



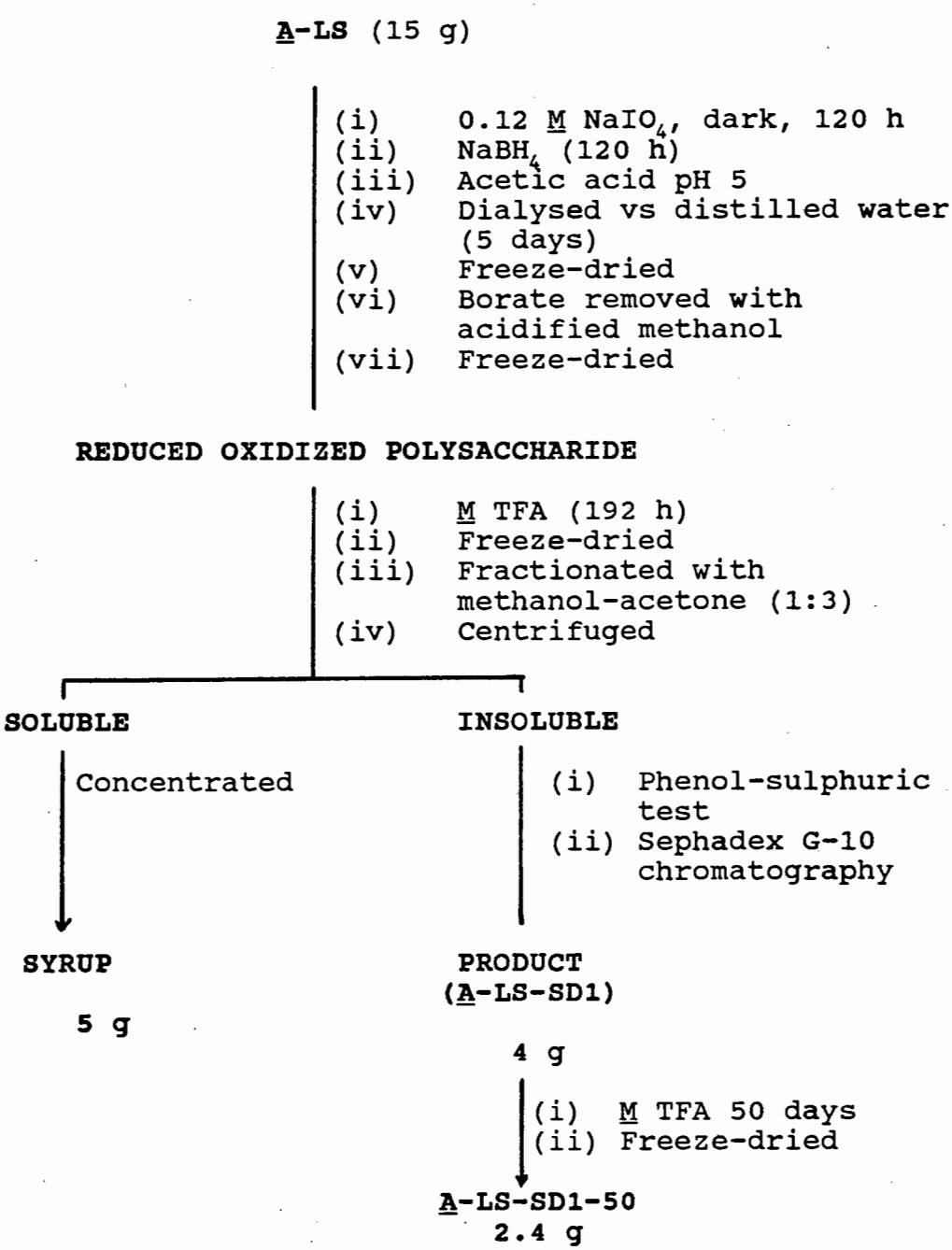
An aliquot of the insoluble product (A-LS) was hydrolysed and derived alditol acetates were analysed by g.l.c. to reveal the presence of xylose, mannose and galactose with only a trace of arabinose (Table 4). Methylation analysis of a portion of A-LS permitted direct comparison with A (Table 5).

### 3.7.4.1 **Smith degradation of A-LS (Scheme 7)**

A sample of A-LS (15 g; Fig. 7.a) was subjected to periodate oxidation with the usual arsenite monitoring. After 120 h the periodate consumption became constant at 7.4 mmol $g^{-1}$, which was not significantly different from that calculated from methylation data (7.6 mmol $g^{-1}$; Table 5).

After borohydride reduction for 120 h, neutralisation and dialysis with suitable monitoring of the dialyzate as before, the solution was freeze-dried. After borate removal the reduced-oxidized product was subjected to mild hydrolysis by M TFA with the usual monitoring by s.e.c. (Bio-Gel P-10). After 192 h the solution was freeze-dried and fractionated by the addition of 1:3 (v/v) methanol-acetone mixture. Centrifugation yielded two products of which the soluble fraction was concentrated under vacuum to yield a syrup (5 g) which on p.c. was found to contain Ara (6+), Xyl (tr) Gal (+) and glycerol (7+).

SCHEME 7 SMITH DEGRADATION OF A-LS



The insoluble fraction, A-LS-SD1 (Table 8) was recovered, dissolved in water and freeze-dried. The phenol-sulphuric acid test indicated that carbohydrate comprised 60% of this fraction. Passage through a column of Sephadex G-10 with water as eluent gave a product (A-LS-SD1; 6 g) which contained glycolaldehyde (4%) and had a wide molecular weight distribution with $M_w$ 6600 (Fig. 7.b).

Prolonged exposure of an aliquot (0.2 g) of this product to M TFA (50 days) resulted in the removal of glycolaldehyde and concomitantly $M_w$ was decreased to 3800 (polydisperse; Fig. 7.c). The molecular weight was monitored weekly by s.e.c. on Bio-Gel P-10 and the reaction was adjudged to be complete when the molecular weight remained constant after two consecutive measurements (Fig. 7.c). This product, A-LS-SD1-50 was subjected to analyses for sugar content, GlcA content and linkage modes. Base degradations with DBU catalyst were performed on the methylated A-LS-SD1-50 and A-LS-SD1 products and also with potassium dimsyl on methylated A-LS-SD1-50 (Table 8).

### 3.7.5 Preparation of the Aldobiouronic Acid 2-O-( β -D-Glucopyranosyluronic acid)-D-Mannose

This experiment was conducted to produce a small quantity of pure aldobiouronic acid (GlcpA-2Man; monomer) which would not only prove the existence of this moiety in the polysaccharide structure of H. sericea, but also allow it to be characterised by the usual methods including $^{13}C$ and $^{1}H$ n.m.r. (1-D and 2-D).

Other components observed present on examination by p.c. were not isolated or further characterised.

After trial experiments to establish the best conditions for isolation of the GlcA-2Man aldobiouronic acid, a vacuum-dried sample of HSLS (5 g) was refluxed at 100°C with 0.05 M sulphuric acid (50 mL) for 20 h. The solution was cooled, then neutralised with barium carbonate, the suspension was centrifuged and the barium sulphate was washed twice with distilled water. Following concentration of the pooled supernatant solutions, the resultant solution was examined by p.c. (solvents B and C) against standard monosaccharides and also an authentic sample of GlcpA-2Man obtained from Encephalartos longifolius gum polysaccharide[30]. The components present were Gal(++), Xyl(+) and Ara(++), and slower-moving components with $R_{Gal}$ values (abundances in parentheses) 0.01 (++), 0.08 (+), 0.26 (+), 0.48 (trace) and

0.61 (+++). The last component had a migration rate equal to that of the monomer. The solution was subjected to chromatography on Sephadex G-10 followed by final separation on Bio-Gel P-10, with water as eluent, to yield GlcpA-2Man (0.02 g) on freeze-drying.

The acid was characterised by the usual methods (Table 11) and by n.m.r.[94].

### 3.7.6 Preparative Partial Hydrolysis of *Hakea sericea* Whole Gum (Scheme 8)

After trial experiments were performed to establish the best conditions for partial hydrolysis of a large sample of polysaccharide to yield the largest number of acidic oligosaccharides, a fresh sample of gum (50 g) was refluxed at 100°C with $H_2SO_4$ (0.5 M, 10 h, 700 mL). The solution was cooled then neutralised with barium carbonate as described and the precipitate was washed with water. Precipitation in ethanol as before yielded two products, an ethanol-soluble product which on concentration gave a syrup (38 g), and an insoluble precipitate which was collected by centrifugation.

Examination of the syrup by p.c. (solvents A, B and C) showed the presence of 5 components: arabinose (4+) with a trace of xylose, galactose (4+), and two oligosaccharides which had $R_{Gal}$ values in solvent C of 0.51 (+) and 0.64 (++) corresponding to the disaccharides Gal-6Gal and GlcpA-2Man.

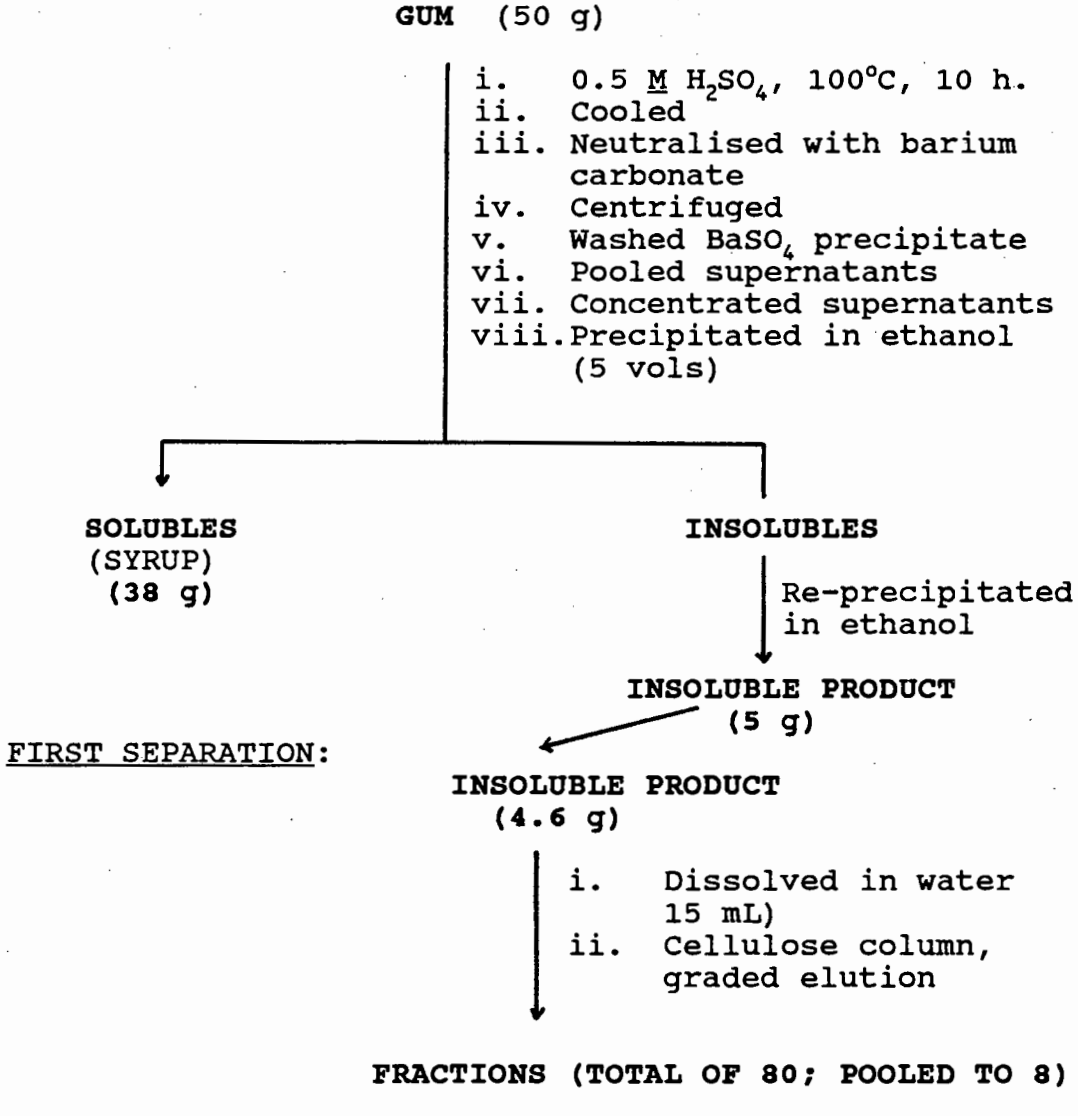
The ethanol-insoluble product was dissolved in water and further purified by re-precipitation with ethanol, collection by centrifugation, re-dissolution in water and freeze-drying, to yield a pinkish, fibrous product (5 g). Examination of aliquots of this latter product on p.c. (solvent B and C)

revealed the presence of at least 6 acidic components with $R_{Gal}$ values (solvent C) of 0.02 (+), 0.08 (+), 0.21 (+), 0.40 (+++), 0.62 (+++) and 0.89 (++). Hydrolysis with 2 M TFA for 18 h followed by examination on p.c. revealed the presence of galactose (++) and mannose (+++). The glucuronic acid content (+++) was determined colorimetrically[85].

The bulk of this product (4.6 g) was dissolved in water (15 mL) and applied to a water-jacketted column (50 x 3 cm) packed with cellulose (Whatman) and maintained at 25°C. Fifty fractions, each of 50 mL, collected every two hours, were obtained on elution with 2:1:1 (v/v) butanol-acetic acid-water at 25 mL/h. The solvent was then changed to aqueous ethanol (15%) and a flow rate of 35 mL/h to give 10 fractions of 70 mL each, and finally changed to water at a flow rate of 35 mL/h, which gave 20 fractions each of 70 mL.

Fractions were examined by p.c.(solvent C) and pooled according to their mobility on paper (p-anisidine spray), Table 12.

SCHEME 8 PARTIAL HYDROLYSIS FOLLOWED BY FIRST AND SECOND COLUMN CHROMATOGRAPHIC SEPARATION OF Hakea sericea WHOLE GUM

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SECOND SEPARATION:

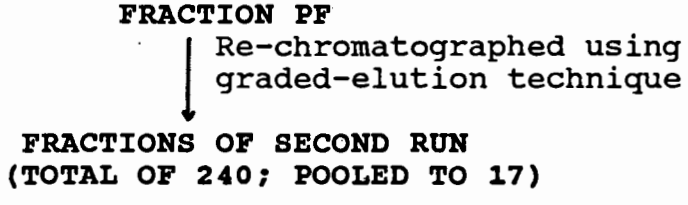


TABLE 12. FRACTIONS COLLECTED DURING THE FIRST CHROMATOGRAPHIC SEPARATION[a] OF THE PARTIALLY HYDROLYSED *Hakea sericea* WHOLE GUM

| POOLED FRACTIONS | WEIGHT(g) | % OF SAMPLE | NUMBER OF COMPONENTS PRESENT[b] |
|---|---|---|---|
| 1 TO 3 | 0.016 | 0.35 | 6 |
| 4 TO 20 | 3.910 | 85.0 | 10 |
| 21 TO 28 | 0.166 | 3.60 | 5 |
| 29 TO 33 | 0.096 | 2.09 | 4 |
| 34 TO 44 | 0.151 | 3.28 | 4 |
| 45 TO 54 | 0.101 | 2.19 | 3 |
| 55 TO 60 | 0.121 | 2.63 | 3 |
| 61 TO 80 | 0.039 | 0.86 | 3 |

a Analysis performed on cellulose column
b According to p.c. results using solvent C.

The separation did not yield any fractions having only one or even two components which would facilitate characterisation. It was thus decided to pool and re-chromatograph the fractions 4 to 20 (hereafter this pooled fraction is referred to as fraction PF) since they were shown to contain most of the aldobiouronic acid GlcA-2Man as well as higher molecular weight substances. The other fractions were retained for analyses at a later stage.

The components of fraction PF, when examined by p.c. (solvent C) alongside the acidic oligosaccharides of Encephalartos longifolius[30] gum, were shown to be of similar mobility to the latter. However, there were no components with mobilities equal to the GlcA-6Gal or higher oligosaccharides containing GlcA-6Gal and 4-MeGlcA-6Gal moieties found in E.longifolius gum. The comparison is shown in Table 13.

These findings stimulated speculation about the similarity in structure between these two gums. The pooled fractions were evaporated to dryness under reduced pressure to yield a product (3.91 g) which was dissolved in water (15 mL) and loaded on to the cellulose column. Graded elution was begun with solvent system butanol-acetic acid-water (6:1:1, v/v) and its composition altered according to the results obtained from p.c. (Table 14). A total of 240 fractions, each collected at 2 hourly intervals, was obtained (see histogram Fig. 10). The fractions were investigated and those with similar components, according to chromatographic mobility (solvents A, B and C) and in certain cases other tests performed at a later stage, were pooled to yield 17 fractions (Table 14).

TABLE 13. COMPARISON OF FRACTION PF OF *Hakea sericea* AND THE ACIDIC COMPONENTS OF THE PARTIALLY HYDROLYSED *Encephalartos longifolius*[a] GUM

| $R_{Gal}$ | COMPONENTS PRESENT IN *H. sericea* | COMPONENTS PRESENT IN *E. longifolius* | DESIGNATION[b] |
|---|---|---|---|
| 0.02 | + | + | A |
| 0.08 | + | + | B |
| 0.22 | + | + | C |
| 0.30 | - | + | D |
| 0.35 | - | + | E |
| 0.42 | + | + | F |
| 0.47 | - | + | GlcA-6Gal |
| 0.65 | + | + | GlcA-2Man |
| 0.80 | - | + | 4MeGlcA-6Gal |
| 0.94 | + | + | GlcpA |
| 1.00 | + | - | Gal |
| 1.23 | tr | - | Xyl |

\+ Present in hydrolysate
\- Absent in hydrolysate
a Acidic oligomers[30]
b According to analysis of *E. longifolius* fractions[30]
A Mainly polymeric GlcA-2Man
B Tetramer of GlcA-2Man
C Dimer of GlcA-2Man
D GlcA-6Gal linked to D-Gal
E $(GlcA\text{-}6Gal)_2$
F Mixture of dimer of GlcA-2Man and trace of GlcA-6Gal

TABLE 14. COLUMN CHROMATOGRAPHY OF FRACTION PF OF *Hakea sericea* GUM USING GRADED ELUTION

| FRACTIONS POOLED | PROPORTIONS OF SOLVENT SYSTEM USED[a] | NEW NUMBER FOR THESE POOLED FRACTIONS |
|---|---|---|
| 19 TO 25 | 6:1:1 | 1 |
| 26 TO 30 | 6:1:1 | 2 |
| 31 TO 35 | 5:1:1 | 3 |
| 36 TO 40 | 3:1:1 | 4 |
| 41 TO 50 | 3:1:1 | 5 |
| 51 TO 54 | 3:1:1 | 6 |
| 55 TO 59 | 3:1:1 | 7 |
| 60 TO 82 | 3:1:1 | 8 |
| 83 TO 93 | 3:1:1 | 9 |
| 94 TO 113 | 3:1:1 | 10 |
| 114 TO 128 | 2:1:1 | 11 |
| 129 TO 145 | 2:1:1 | 12 |
| 146 TO 149 | 2:1:1 | 13 |
| 150 TO 154 | 2:1:1 | 14 |
| 155 TO 171 | 2:1:1 | 15 |
| 172 TO 179 | 20% aq.ethanol | 16 |
| 180 TO 240 | water | 17 |

a Solvent systems composed of butanol : acetic acid : water mixed in proportions of decreasing butanol content from 6:1:1 to 2:1:1.; thereafter aqueous ethanol and finally water were used for elution.

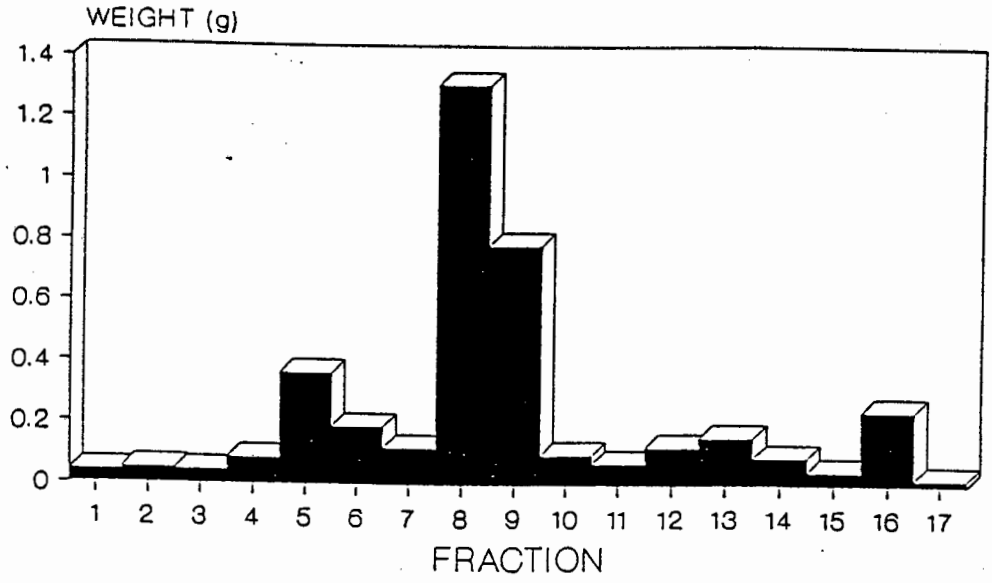



FIGURE 10. FRACTIONATION OF FRACTION PF OF Hakea sericea - THE HISTOGRAM ILLUSTRATING COLUMN PERFORMANCE

Prior to analyses, all fractions were co-evaporated with water to dryness, dissolved in water and collected by freeze-drying.

In the investigation of each of these fractions all paper chromatographic separations were performed in solvents A, B, and C and authentic substances were used in the designations of components wherever possible; all values for GlcA quoted for fractions were determined using the Blumenkrantz method[85].

Fraction 1: Weight 0.04 g. Isolated as a brown syrup, $R_{Gal}$ (solvent C) 2.01. Chromatography with standards and also co-chromatography with the authentic specimens showed this fraction to be composed mainly of glucuronic acid, with a trace of D-glucurono-3,6-lactone.

Fraction 2: Weight 0.05 g. A crystalline product which on p.c. examination was shown to be composed of arabinose and xylose. Hydrolysis followed by g.l.c. (column B) of the alditol acetates showed the presence of arabinose (80%) and xylose (20%), i.e. 0.04 g and 0.01 g respectively.

Fraction 3 : Weight 0.045 g. This crystalline fraction on p.c. and g.l.c. (column B) was shown to be composed of galactose (83% ; 0.037 g) and mannose (17%; 0.008 g).

Fraction 4: Weight 0.086 g. A crystalline solid with $[\alpha]_D$ + 6.8° (c 1.0) which on examination by p.c. was shown to contain galactose only. Unambiguous identification was made from m.p., g.l.c. (column B) and $^1H$ n.m.r. (200 MHz) analyses.

Fraction 5: Weight 0.365 g. A white crystalline product, $[\alpha]_D$ + 3.1° (c 1.46) composed mainly of glucuronic acid, with a trace of galactose.

Fraction 6: Weight 0.190 g. A white crystalline product with $[\alpha]_D$ + 5.0° (c 1.32) which on examination by p.c. and $^1H$ n.m.r.examination showed the presence of glucuronic acid only.

Fraction 7: Weight 0.121 g. An off-white crystalline product $[\alpha]_D$ -1° (c 1.4) which was examined by p.c. versus standards and co-chromatography with authentic specimens. Hydrolysis and g.l.c. (column B) of the derived alditol acetates showed the presence of Gal (6%), Man (38%) and glucose (56%), the glucose arising from reduction of GlcA with $NaBH_4$. These approximated the p.c. results allowing for incomplete reduction of all the glucuronic acid.

All these results indicated this fraction to be composed of glucuronic acid (++), galactose (+) and 2-O-(β-D-glucuronopyranosyl)-D-mannose (+).

Fraction 8: Weight 1.314 g. A whitish product $[\alpha]_D$ -34° (c 1.23), uronic acid 50%, $R_{Gal}$ 0.60 (solvent C) [ochre coloured spot on interaction with p-anisidine HCl spray] and mol.wt. 360 (Bio-Gel P-2). Hydrolysis in 2 M TFA for 18 h followed by p.c. examination revealed the presence of mannose mainly, with a trace of glucuronic acid.

Methylation of this component gave a product, $[\alpha]_D$ -36.6° (c 2.0) which on hydrolysis and g.l.c. analysis (column B) showed the presence of only 3,4,6-Man. LAD reduction of this fraction gave a product, $[\alpha]_D$ -20° (c 1.1) which on hydrolysis and g.l.c. (column B) gave 2 components, 3,4,6-Man and 2,3,4-Glc, in a ratio of 1:1, indicating the presence of glucuronic acid only as the non-reducing end-group and

mannose only as the 2-linked form. 1-D and 2-D n.m.r. methods using $^{1}H$, APT, $^{13}C$, HETCOR and COSY variations confirmed these results.

Examination by p.c. against authentic substances and co-chromatography with authentic 2-O-( β -D-glucopyranosyluronic acid)-D-mannose (ex E.longifolius gum[30]; solvent C) gave results indicating the presence of only 1 component, corresponding to this authentic specimen, with $R_{Gal}$ 0.60.

Positive f.a.b.-m.s. of this permethylated fraction gave a spectrum (Fig. 11) which showed peaks for the cationised molecular ions, $[M + NH_4]^+$ and $[M + Na]^+$ at m/z values of 486 and 491.

Fragment ion m/z 405 is probably the result of the loss of 2 molecules of methanol from the protonated molecular ion $[M + 1]^+$ m/z 469. Other ions seen on the spectrum are listed :

| permethylated component | m/z |
|---|---|
| $A^+$ -MeOH | 201 |
| $A^+$ | 233 |
| $AM^+$ | 437 |

$A^+$ = GlcA

Indications exist for the adherence of fragmentation of the

monomer (3) to the Pathway A cleavage, viz. glycosidic bond cleavage[79].

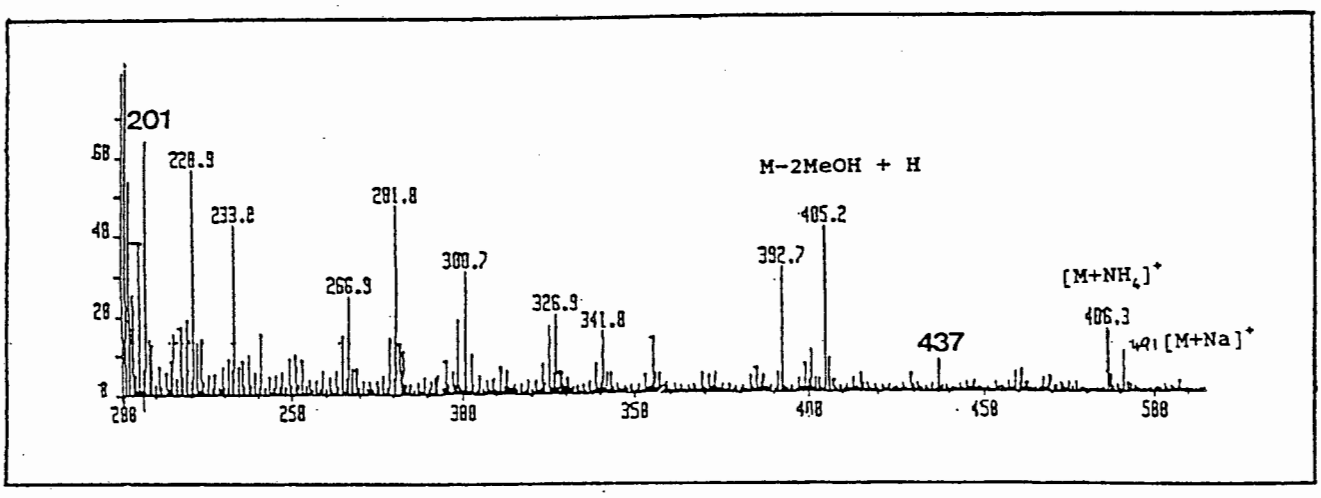



FIGURE 11. POSITIVE F.a.b.-m.s. of GlcA-2Man, 3.

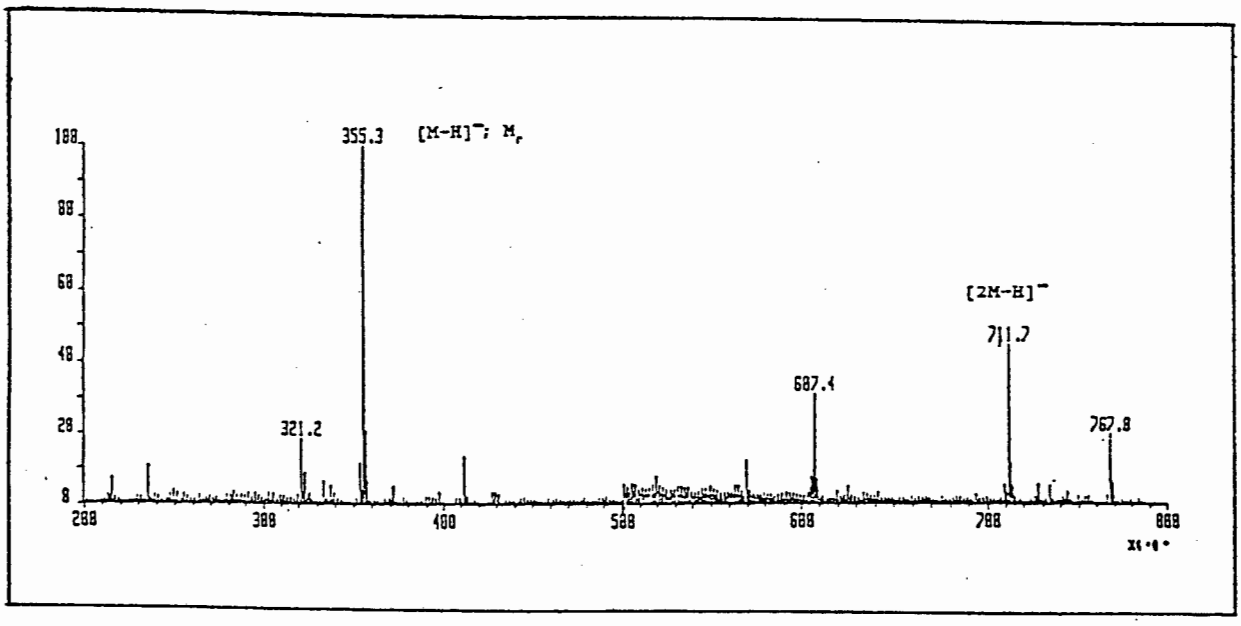



FIGURE 12. NEGATIVE F.a.b.-m.s. of GlcA-2Man, 3.

Negative f.a.b.-m.s. of the native monomer (Fig. 12) accurately indicated the molecular weight of the monomer viz. 356 (molecular ion, $M_r$) made up of m/z values 176 (GlcA), 162 (Man) and 18 ($H_2O$).

The formation of an ionisation dimer formed in the ionisation chamber was evidenced by the peak of m/z value 711 [2M-H], formed by combination of 2 molecules of the monomer minus 1 proton. Sometimes underivatised carbohydrates exhibit intense "minus 2" signals as a result of oxidation. Other peaks unexplained at present include those at 411, 607, 767, 847 and 1203 (all magnified 4 times on the spectrum). Generally fragmentation patterns are not easily predictable in negative f.a.b.-m.s. of underivatised oligosaccharides[79].

Fraction 9 : Weight 0.787 g. A white fluffy product with $[\alpha]_D$ -31.5° (c 1.0). P.c. (solvent C) showed the presence of two spots (components), one at $R_{Gal}$ 0.60 (9+) (identical in its ochre colour with fraction 8) and the other at $R_{Gal}$ 0.38 (+) [a brown colour with p-anisidine HCl spray]. P.c. examination (solvent B) showed a spot at the origin (3+) and another at $R_{Gal}$ 0.10 (tr). The molecular weight distribution on s.e.c. (Bio-Gel P-2) was 440, 360, ratio 1:3.
Hydrolysis followed by g.l.c. of the prepared alditol acetates revealed the presence of galactose 4%, mannose 66% and glucose 30%. The last sugar was not present on p.c. of the acid hydrolysate.

Preparative p.c. (solvent C) of 0.2 g yielded a product with $R_{Gal}$ 0.60 (0.18 g) and another with $R_{Gal}$ 0.38 (0.010 g). The former product was analysed using the same methods as were applied to fraction 8, and the results obtained showed that this fraction was identical to fraction 8, i.e. GlcpA-2Man (3).

The oligosaccharide having $R_{Gal}$ 0.38, was shown on examination by p.c. (solvent C) to be composed of Gal (+) and Man (+). GlcA content was determined to be 30%. Methylation analysis including LAD reduction revealed the presence of GlcA-, 4,6-Man (+) and 2,3,4,6-Gal (tr). A probable structure for this oligosaccharide is GlcA-2Man3-Gal (12).

Fraction 10: Weight 0.098 g. A white fluffy product, $[\alpha]_D$ +9° (c 1.33). Molecular-weight distribution (Bio-Gel P-2) was 660, 550, and 460, ratio 1:1:1 . P.c. (solvent C) showed the presence of 2 components, at $R_{Gal}$ values 0.28 (+) and 0.38 (+). This fraction did not migrate from the origin of the paper chromatogram after 36 hours in solvent B.

Hydrolysis in 2 M TFA for 18 h followed by p.c. (solvent C) revealed the presence of GlcA-2Man (tr), mannose (++) and Gal (+). G.l.c. of the prepared alditol acetates gave Man 70% and Gal 30% (column A).

This fraction was thus shown to be a mixture of acidic oligosaccharides of $R_{Gal}$ 0.38 (as in fraction 9) and $R_{Gal}$ 0.28 (see fraction 11).

Fraction 11 : Weight 0.07 g. A white fluffy product, $[\alpha]_D$ + 1.6° (c 0.66), molecular-weight distribution (Bio-Gel P-2) of 730 and 460, in ratio 1:2.

Examination by p.c. (solvent C), however, revealed the presence of only one component at $R_{Gal}$ 0.28 (which had the same mobility as one of the two components in fraction 10 when co-chromatographed on paper in this solvent. $R_{Gal}$ was 0.53 on p.c. in solvent D and the spot remained at the origin on p.c. in solvent B.

Hydrolysis followed by p.c. and g.l.c. (column A) gave the following results Man 50%, Gal 20%, while the GlcA (30%) was determined colorimetrically. Partial hydrolysis of 5 mg in 0.5 M TFA at 100°C for 3 h followed by p.c. (solvent C) showed the presence of GlcA-2Man (+), Man (+) and Gal (tr). Methylation analysis and LAD reduction followed by g.l.c. and g.l.c.-m.s. revealed the presence of the following sugars: 2-linked Man (1), 4-linked GlcA (0.7), GlcA- (1.0), 6-linked Galp (0.4) and 6-linked Galf (tr). $^1H$ n.m.r. showed signals at $\delta$ 5.40 , 4.99 (1H) , 4.56 ($J_{1,2}$ 8Hz, 1H), and 4.49 ($J_{1,2}$ 8Hz, 1H), also a coalesced signal $\delta$ 5.21 to 5.26, (Table 15).

TABLE 15. $^1$H-N.M.R. DATA (200 MHz) OF FRACTION 11

| CHEMICAL SHIFT (p.p.m). | POSSIBLE ASSIGNMENT OF ANOMERIC PROTONS |
|---|---|
| 5.40 | -2Man α - |
| 4.99 | -2Man β -OH |
| 4.52 | $Glc_pA$ β - |
| 4.49 | -4GlcA β- |

Due to the paucity of this fraction no further analysis could be performed. A number of permutations of structure are possible for these components. The monomer was present in this tetrasaccharide structure but whether the monomer had joined to it 4-linked GlcA or 6-linked galactose remains unsure. This fraction could be GlcA-6Gal-4GlcA-2Man (**13**) or GlcA-2Man-4GlcA-6Gal (**13.a**). The former structure seems to be favoured by the n.m.r. evidence of -2Man β-OH at the reducing end while the only evidence for -6Gal at the reducing end is the trace amount of -Gal*f* found on g.l.c.-m.s. Further study is indicated at a later stage when the oligosaccharide is available.

Fraction 12 : Weight 0.124 g. A white fluffy product with $[\alpha]_D$ -12° (*c* 0.66); mol.wt. 760 (Bio-Gel P-2); p.c.: showed a spot at $R_{Gal}$ 0.25 (solvent A), only a spot at the origin (solvent B) and $R_{Gal}$ 0.53 (yellow colour, solvent D). Hydrolysis in 2 *M* TFA followed by p.c. (solvent C) showed

the presence of GlcA-2Man (+), GlcA (+)], Gal (tr), Man (++), glucuronolactone (tr). G.l.c. of the alditol acetates gave Man 58% and Gal 2%. GlcA (40%) was determined by the method of Blumenkrantz[85]. Partial hydrolysis (5 mg) in 1 M TFA for 40 minutes followed by p.c. (solvent C) gave spots for the original (++) and products GlcA-2Man (4+), Gal (+), Man (tr), glucuronolactone (+). Methylation analysis showed only 2-linked Man (84%) and 6-linked Gal (14%). LAD reduction of the methylated fraction followed by g.l.c-m.s. (column A) showed the presence of 2-linked Man (33%), GlcA- (32%), 6-linked Gal (7%) and 4-linked GlcA (28%). The $^1$H n.m.r. spectrum showed signals at $\delta$ 5.39 (s, 1.0 H), 5.27 (s, 0.8 H), 4.96 (s, 0.2 H), 4.53 [ $J_{1,2}$ 8Hz, 1H],and 4.49 [$J_{1,2}$ 8Hz, 1H]. Results are presented in Table 16.

TABLE 16. $^1$H-N.M.R. DATA (200 MHz) OF FRACTION 12

| CHEMICAL SHIFT (p.p.m.) | DESIGNATION OF ANOMERIC PROTONS |
|---|---|
| 5.39 | -2Man $\alpha$ - |
| 5.27 | -2Man $\alpha$ -OH |
| 4.96 | -2Man $\beta$ -OH |
| 4.53 | GlcA $\beta$ - |
| 4.49 | -4GlcA $\beta$- |

The results obtained in the study of this fraction indicate that it is composed of GlcA-6Gal-4GlcA-2Man or, less likely, because of the mol.wt. and lack of a linkage residue, the dimer (**14**) of GlcA-2Man substituted with a unit of 6-linked galactose.

Fraction 13 : Weight 0.155 g. A white fluffy product with $[\alpha]_D$ -17.8° (c 1.0), mol.wt. 720. P.c. (solvent C) gave one spot with $R_{Gal}$ 0.25; $R_{Gal}$ was 0.51 in solvent D and the spot remained at the origin on p.c. in solvent B. GlcA content was 50% and hydrolysis with 2 M TFA for 18 h, followed by p.c. (solvent C) showed the presence of GlcA-2Man (+), GlcA (+) and Man (+++). G.l.c. of the prepared alditol acetates indicated the presence of Man (66%) and Glc (34%; the glucose again originated from the GlcA of this component).

Methylation analysis and LAD reduction followed by hydrolysis and g.l.c. and g.l.c.-m.s. examination gave products with the following data:

(i) a methylated product with $[\alpha]_D$ -22° (c 2.4) which on hydrolysis followed by p.c. examination gave 2 spots coinciding with authentic methylated GlcA and 3,4,6-Man.

(ii) a LAD-reduced product, $[\alpha]_D$ -9° (c 1.00) which on hydrolysis followed by p.c. and g.l.c.-m.s. was found to contain 2-linked Man (50%), 4-linked GlcA (21%) and non-reducing end-group GlcpA (29%).

This fraction appears to consist entirely of the dimer (**14**) of GlcA-2Man. This was confirmed by n.m.r. analysis of the 1-D and 2-D types, utilising $^1H$, $^{13}C$, APT, COSY and HETCOR methods, and further evidence was obtained from f.a.b.-m.s. of the permethylated product (see Section 3.7.6.).

Fraction 14 : Weight 0.089 g. A white fluffy product, $[\alpha]_D$ -13.1° (c 0.76), with GlcA content 48%. P.c. (solvent C) gave one spot at $R_{Gal}$ 0.23. Hydrolysis followed by p.c. and g.l.c. of prepared alditol acetates gave the following sugars : GlcA-2Man (+), GlcA (+), Gal (tr), Man (++++), glucuronolactone (+), and Glc (+). The Glc originated from the GlcA when these units were reduced by $NaBH_4$.

The values obtained from g.l.c. analysis (column A) of the derived alditol acetates were Gal 3%, Man 71% and Glc 26%. The $^1H$ n.m.r. spectrum showed signals with chemical shift values identical to those of fraction 13 (see Section 3.7.6). The trace of Gal did not give any significant signal on the $^1H$ n.m.r. (200 MHz) spectrum.

This fraction is composed of the dimer with a trace of galactose as an "impurity" not bound to the structure.

Fraction 15 : Weight 0.044 g. The product has $[\alpha]_D$ +8.5° (c 0.66), $\bar{M}_w$ 1050 (Bio-Gel P-2). P.c. showed one component at $R_{Gal}$ 0.15 (solvent C). Sugar composition was shown to be Gal 12%, Man 45%, GlcA 43%, and partial hydrolysis with 0.5 M TFA at 100°C for 3 h followed by p.c. (solvent C) of the freeze-dried residue yielded the dimer of GlcA-2Man (+), monomer of GlcA-2Man (+), Gal (+), Man (tr).

Methylation analysis including LAD reduction followed by g.l.c. and g.l.c.-m.s. showed the presence of 2-linked Man (42%), 6-linked Gal (15%), 4-linked GlcA (30%), and non-reducing end-group GlcA (13%). No branch points were detected.

$^1$H-n.m.r. (200 MHz) results are presented.

TABLE 17. $^1$H-N.M.R. DATA (200MHz) OF FRACTION 15

| CHEMICAL SHIFT (p.p.m.) | ASSIGNMENT | INTEGRATION |
|---|---|---|
| 5.40 | -2Man α - | 2H |
| 5.28 | -2Man α -OH | 0.8H |
| 4.96 | -2Man β -OH | 0.2H |
| 4.53 | GlcpA β - | 1H |
| 4.49 | -4GlcpA β - | 1H |

The results indicate that this fraction could be a structure composed of approximately seven sugar units including a dimer and a monomer in equal amounts separated by an acid-susceptible galactose moiety.

The results indicate this fraction to be composed of dimer, 6-linked Gal and the monomer possibly as dimer-6Gal-monomer **(15)**.

Fraction 16: Weight 0.241 g. A white fluffy product with $[\alpha]_D$ +4.2° (c 1.36) and molecular-weight distribution of 1600, 1350, 1100, 660, 540, 504 and 430, with $\bar{M}_w$ 1300. P.c. (solvent C) showed a component at $R_{Gal}$ 0.07 which remained at the origin on p.c. in solvent B. Sugars were shown to be Gal 25%, Man 39% and GlcA 36%.

Partial hydrolysis was conducted using various conditions to remove only Gal but not any Man. This was monitored by p.c. (solvent C) which indicated the presence of the dimer, monomer and disaccharide Gal-6Gal in the hydrolysate (Table 18).

TABLE 18. PARTIAL HYDROLYSIS OF FRACTION 16 IN 0.5 M TFA OVER DIFFERENT PERIODS OF TIME

| $R_{Gal}$ | Period of exposure to 0.5 M TFA (minutes) | | | Designation |
|---|---|---|---|---|
| | 30 | 60 | 90 | |
| 0.06 | + | + | + | Fraction 16 |
| 0.21 | - | + | <+ | Dimer |
| 0.24 | ++ | - | - | ? |
| 0.29 | - | + | <+ | ? |
| 0.49 | + | + | + | Gal-6 Gal |
| 0.60 | + | + | ++ | Monomer |
| 1.00 | - | ++ | +++ | Gal |
| 1.28 | tr | tr | tr | Glucurono-lactone |

Ion-exchange column chromatography on Bio-Rad AG1X2 $F^-$ (column dimensions 20 x 1 cm) was used to separate the products of the following partial hydrolysis:

Fraction 16 (90 mg) was refluxed for 30 minutes in 0.5 M TFA (2 mL), which was then removed by co-evaporation with water. The dry residue was dissolved in water and 1 mL charged to the column. Neutral components were eluted by water and the acidic components by 20% formic acid. Monitoring was performed by p.c. (solvent C) versus standards and also co-chromatography with the authentic specimens (Table 19).

TABLE 19. THE PRODUCTS OF PARTIALLY HYDROLYSED FRACTION 16, THEIR CONCENTRATIONS AND DESIGNATIONS

| $R_{Gal}$ values | RELATIVE CONCENTRATIONS | DESIGNATION |
|---|---|---|
| **NEUTRAL COMPONENTS:** | | |
| Origin | ++ | Fraction 16 |
| 0.23 | + | Dimer |
| 0.35 | + | ? |
| 1.00 | ++ | Gal |
| 1.35 | tr | Man |
| 1.5 | tr | Glucuronolactone |
| **ACIDIC COMPONENTS:** | | |
| Origin | + | Fraction 16 |
| 0.23 | + | Dimer |
| 0.64 | ++ | Monomer |

$^1$H-n.m.r.(200 MHz) spectrum showed signals at chemical shift values 5.39 (2H) corresponding to -2Man $\alpha$ -, 5.26 (0.75 H) and 4.96 (0.25H) characteristic of the $\alpha$ - and $\beta$ - forms of end group -2Man-OH, with signals at $\delta$ 4.52 and 4.49 indicative of GlcpA-. In-chain GlcA was present but was not as clearly indicated as in the dimer.

Methylation analysis including LAD reduction were performed. The results obtained for the alditol acetates of the LAD-reduced methylated fraction on g.l.c.-m.s. (column B) are presented here.

| COMPONENTS | MOL % |
|---|---|
| T-Gal | 10 |
| -2 Man | 30 |
| GlcpA | 12 |
| -4 GlcA- | 30 |
| -6 Gal | 9 |
| -2,3 Man | 9 |

This fraction (**16**) would seem to be composed of a trimer (i.e. three monomers) with the penultimate mannose being substituted at its 3 position by a disaccharide Gal-6Gal.

Fraction 17 : Weight 0.019 g. A white crystalline substance $[\alpha]_D$ +2.7° (c 0.66) composed of Gal only. The Blumenkrantz method showed that GlcA was absent. Hydrolysis followed by g.l.c. of the prepared alditol acetates confirmed that Gal was the only sugar component in this fraction. On p.c. (solvent C) this fraction did not move from the origin. No further analysis was possible due to the paucity of the sample.

The other seven fractions obtained from the first chromatographic run were examined by p.c. (solvents B and C) against Gal and components obtained from the second chromatographic run, viz. monomer, dimer and other slower migrating oligosaccharides, Table 20.

TABLE 20. RESULTS OF PAPER CHROMATOGRAPHY OF FRACTIONS OBTAINED FROM THE FIRST CHROMATOGRAPHIC PROCEDURE ON CELLULOSE

| | FRACTION NUMBERS | | | | | | |
|---|---|---|---|---|---|---|---|
| $R_{Gal}$ | 1-3 | 21-28 | 28-33 | 34-44 | 45-54 | 55-60 | 61-END |
| 0.06 | + | + | + | + | + | + | + |
| 0.24 | + | + | + | + | + | - | - |
| 0.38 | + | + | + | + | + | - | - |
| Gal | + | + | + | + | + | + | + |

The oligosaccharides: $R_{Gal}$ 0.06 had a migration equal to that of fraction 16, $R_{Gal}$ 0.24 moved at the same rate as the dimer and the remaining component with $R_{Gal}$ 0.38 was probably the component present as one of two oligosaccharides in fraction 9.

### 3.7.6.1 Spectroscopic evidence for the structure of the dimer of GlcA- 2Man

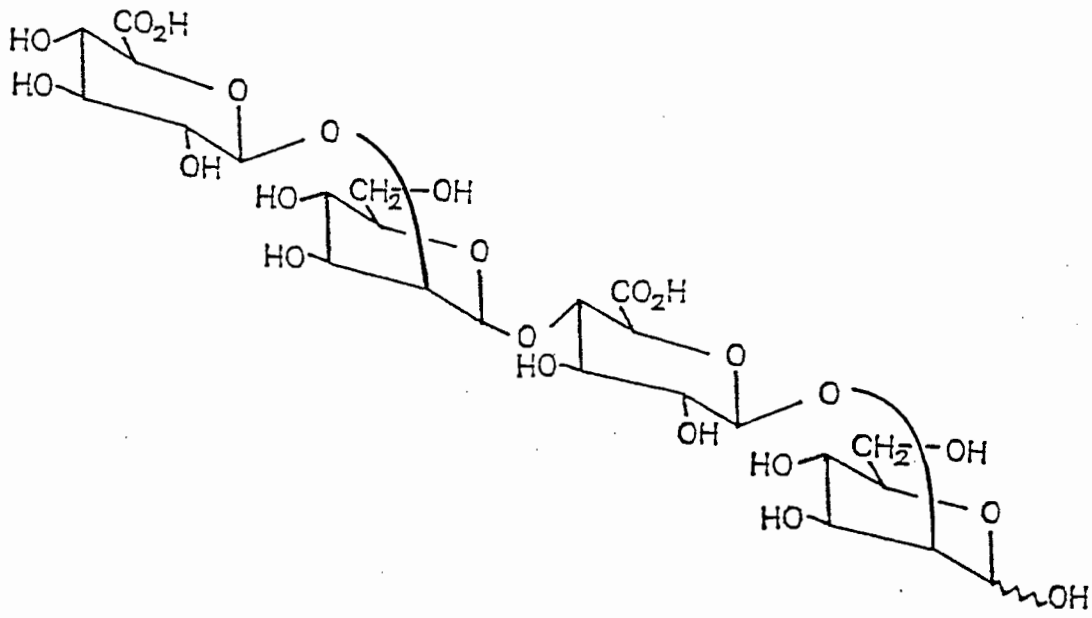
#### 3.7.6.1.1 N.m.r. analyses of 14

The tetrasaccharide in fraction 13 (dimer; structure **14**), was characterised by the use of 1D- and 2D-n.m.r. spectroscopic methods. In this study the general strategy used to assign the signals of the components of the dimer was the following: comparisons with published chemical shift data for model compounds (mentioned below), especially the values for their anomeric proton signals which were used as starting points in the interpretation of the results of the COSY experiment. This exercise did not lead to complete assignment of all the protons, but those signals that were identified were used in the interpretation of the results of the HETCOR and $^{13}C$-n.m.r. experiments, to complete the study.

Use was made of the following model compounds :

(i) monosaccharides α- and β-D-Man-OH ,and β-D-GlcpA[71]
(ii) the monomer, viz 2-O-β-D-glucuronopyranosyl-D-mannose[94] [which during the course of this study was isolated from both H. sericea and H. gibbosa gums],
(iii) the dimer from Chorisia speciosa[17] polysaccharide,

(iv) the trimer (OTBT) and the polymer (OTB), both products of partial hydrolysis of the polysaccharide from Ornithogalum thyrsoides[37] (OT), which were composed of linear sequences of alternating β -D-glucuronic acid and D-mannose molecules i.e. $(GlcA\text{-}2Man)_n$; the polymeric product was retained by dialysis tubing with molecular weight cut-off of 3000, and was precipitable in ethanol (5 vols).



**14**

The anomeric proton shifts and coupling constants of the dimer, reported by Dutton[17], closely resembled those values obtained in this study of the dimer (Table 21 and Fig. 13).

TABLE 21. ANOMERIC PROTON CHEMICAL SHIFTS OBTAINED FROM $^1$H-N.M.R. (200 MHz) ANALYSIS OF THE ACIDIC TETRASACCHARIDE (DIMER; **14**) FROM Hakea sericea GUM

| CHEMICAL SHIFTS $\delta^a$ | $\delta^b$ | INTEGRAL PROTON[a,b] | ASSIGNMENT |
|---|---|---|---|
| 5.40 | 5.42 | 1.0 | -2Man α - (**E**)[c] |
| 5.29 | 5.27 | 0.8 | -2Manα-OH (**F**)[d] |
| 4.98 | 4.96 | 0.2 | -2Manβ-OH (**F**)[d] |
| 4.53 | 4.55 | 0.80[b](1.0 H[a]) | GlcAβ- (**C**)[e] |
| 4.49 | 4.49 | 1.0 | -4GlcAβ- (**E**)[f] |

$\delta^a$ and $\delta^b$ refer to the signals of the dimers from Chorisia speciosa gum[17] and H. sericea respectively, both of these derived chemical shifts being relative to internal acetone at δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate.
a and b refer to the integral proton for C. speciosa dimer[17] and H. sericea dimer respectively.
c,d,e and f Units as designated in Table 22

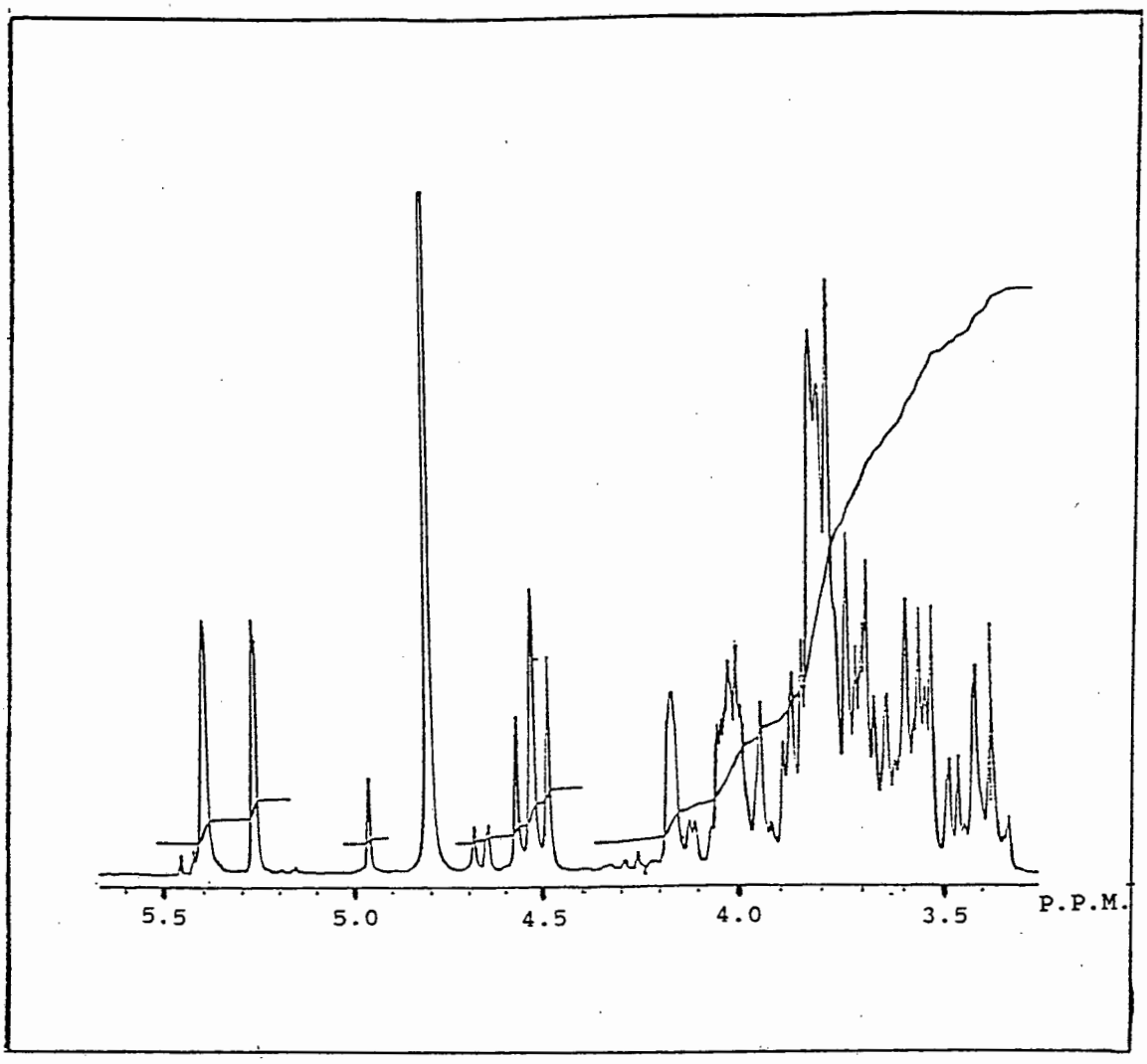



FIGURE 13 $^{1}$H-N.M.R.(200 MHz) OF **14** RECORDED AT 25$^{0}$C

It was decided to focus on the elucidation of the Man and GlcA end groups of the dimer since these had already been examined as a combination in the disaccharide (monomer; GlcA-2Man) from H. sericea gum[94]. Morover their values would be comparable with those of the free sugars[71]. By subtraction, the component carbons and protons of the in-chain groups could then be identified.

In the analysis of the monomer[94] the α - and β -Manp were shown to be present by the anomeric proton signals at δ 5.27 (0.75H, $J_{1,2}$ = 1.4Hz) and δ 4.96 (0.25H, singlet) respectively, while in the $^1$H-n.m.r. spectrum of the dimer of H. sericea gum (Fig. 13) the only difference for the reducing end group Manp was the increased α : β ratio of 4:1 which was greater than the values quoted for the free sugar (66%)[97] and the monomer (75%). The reason for this change upon O-2 substitution of the mannose has been rationalised in terms of the increased anomeric effect[97].

The use of the anomeric proton of the β -Man end group allowed the unambiguous assignment of the H-2 signal but for α -Man utilisation of the spin systems shown in the COSY spectrum (Fig. 14) allowed identification of H-2 and H-3 at 4.03 and 3.80. Further accurate assignments for the α -Man end group and other residues of the dimer were limited to H-2 identification (Fig. 14). However these proton assignments allowed the establishment of certain C,H correlations using the HETCOR experiment and gave values for the C-2 of β -Man at δ 81.19, and C-2 and C-3 of α Man at δ 79.18 and 70.18 respectively (Fig. 15).

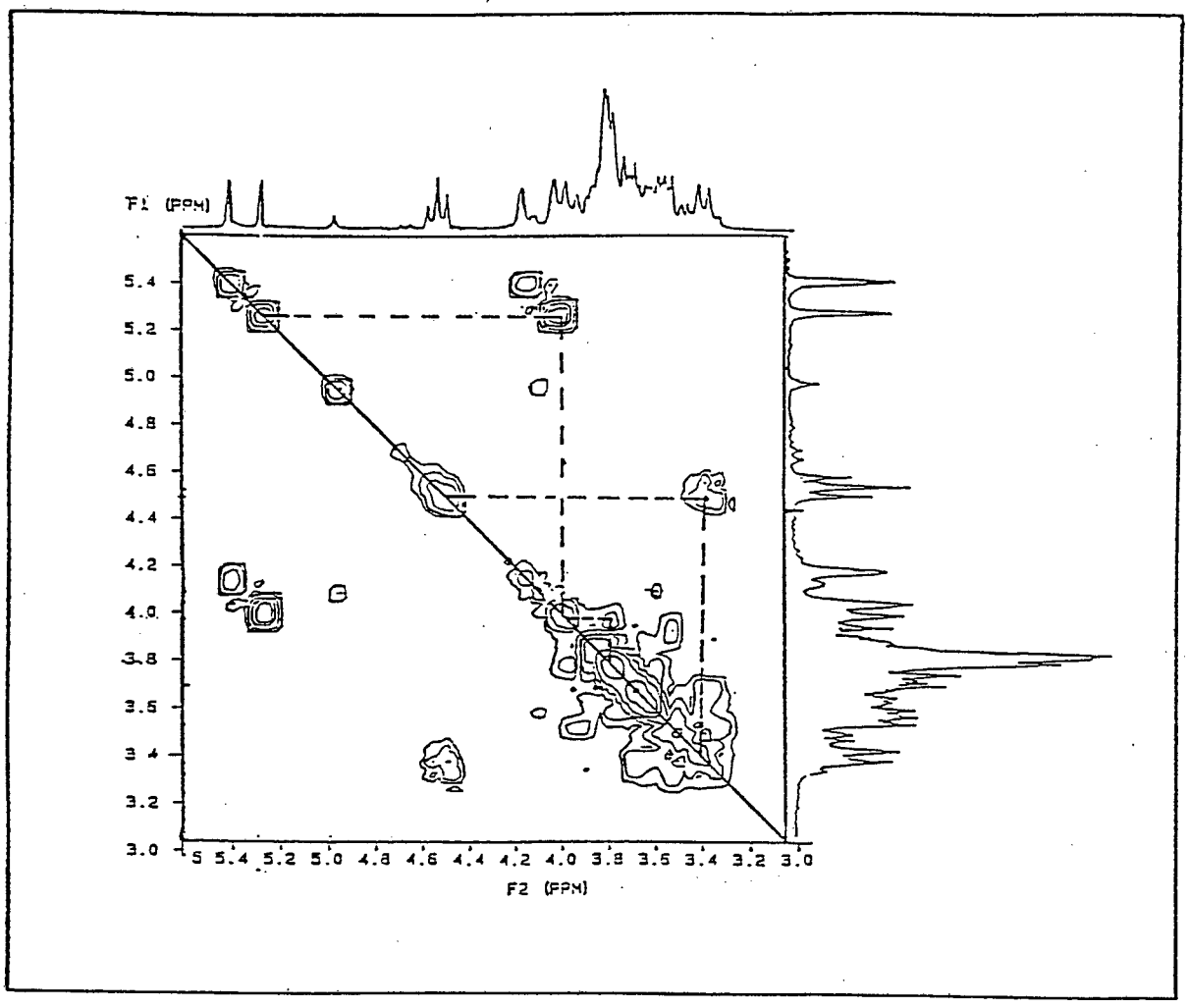



FIGURE 14. COSY SPECTRUM OF **14** RECORDED AT 25°C; SOME OF THE SPIN SYSTEMS ARE SHOWN

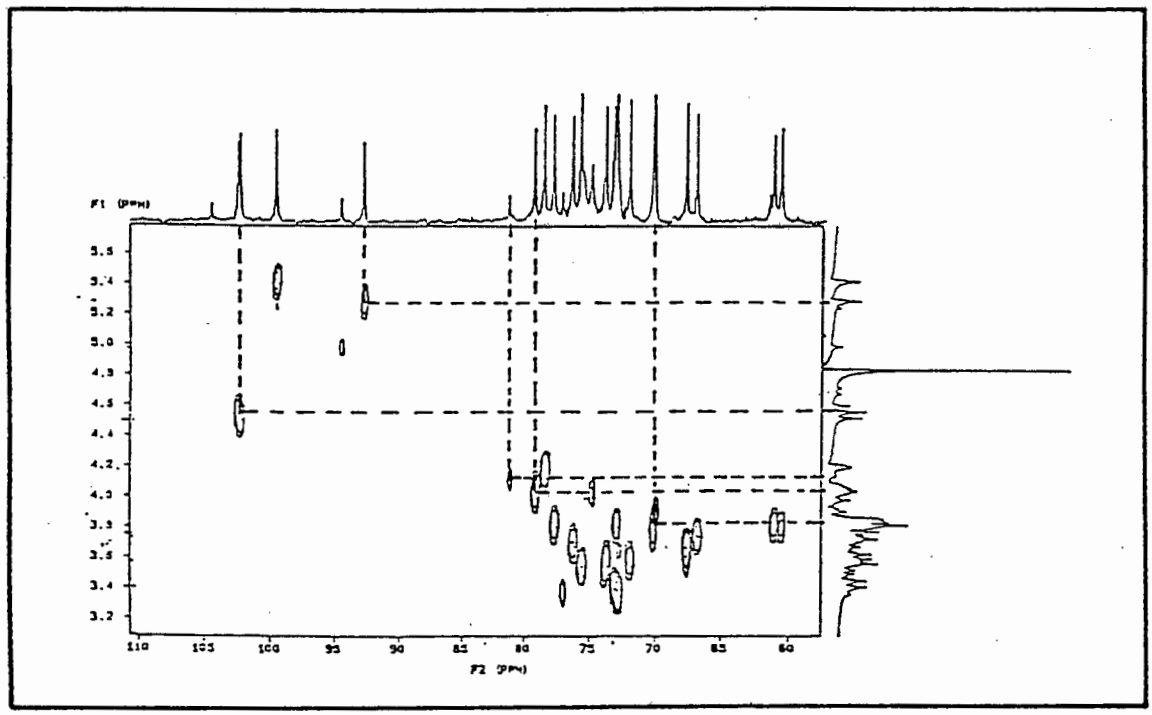


FIGURE 15. HETCOR SPECTRUM OF **14** RECORDED AT 25°C; SOME OF THE C,H CORRELATIONS ARE SHOWN

Further $^{13}C$ assignments (spectrum as Fig. 15) of the end group Manp ( α and β forms) were made after comparison with the free sugars[71] and the monomer[94] (utilising the rules of Bradbury and Jenkins)[71]. Here the α - and β-forms of Manp were easily distinguished from each other on the basis of their signal intensities where 1 α:1 β = 3:1. The $^{13}C$ resonances of the three C-6 carbon atoms of the mannose residues i.e. the two end groups and in-chain, were easily identified by their behaviour in the APT experiment since they are the only

methylene carbons in these residues; the α : β anomers of Man end group were also identified using the rule of best fit relative to the model compound, free mannose[71]. The signal due to the C-6 of the β-Man was of low intensity which did not allow its connectivity with H-6 to be established by the HETCOR experiment. The results are presented in Tables 22 and 23.

Proton assignments for **14** were deduced from the COSY and HETCOR experiments. In the non-reducing end-group GlcpA (**C**) of the dimer (**14**), H-1 ( δ 4.55) was assigned by comparison with the C. speciosa $^{1}$H-n.m.r. spectrum, and the $^{13}$C spectrum allowed assignments of the carbons of the GlcpA to be made using the previously mentioned approach for end-group mannose. As a result, confirmation of the chemical shift allocation for the anomeric proton was obtained from the HETCOR experiment.

H-2 was then assigned by the use of the COSY experiment and verified by the $^{13}$C results. Further assignments of protons and carbons were accomplished by the use of the HETCOR experiment. The large glycosylation shift differences (Table 23) for C-1 and C-2 of α- and β-Man , and C-1 of GlcpA, from those of the model compounds confirm the sites of linkage[71].

TABLE 22. $^{13}C$ RESONANCES OF THE MANNOSE END-GROUP AND GlcpA END-GROUP OF THE DIMER FROM Hakea sericea GUM (IN $D_2O$) AT 25°C.

Compounds referred to in this Table :

The monomer (3) : β -D-GlcA-(1-2)-α , β -D-Man-OH ,
**A** **B**

and the dimer (14) :
β -D-GlcA-(1-2)- α -D-Man-(1-4)- β -D-GlcA-(1-2)-α , β -D-Man-OH
**C** **D** **E** **F**

| END GROUP | CHEMICAL SHIFT (p.p.m.) | | | | | |
|---|---|---|---|---|---|---|
| | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| REDUCING END GROUP : | | | | | | |
| α -Manp(**F**) of dimer | 92.72 | 79.18 | 70.18 | 67.68 | 73.22 | 61.20 |
| **Glycosylation shift**[a] | **-2.25** | **+7.49** | **-1.17** | **-0.36** | **-0.14** | **-0.93** |
| α -Manp(**B**) of monomer | 92.77 | 79.39 | 70.15 | 67.74 | 73.06 | 61.23 |
| **Glycosylation shift**[a] | **-2.2** | **+7.7** | **-1.2** | **-0.3** | **-0.3** | **-0.9** |
| β -Manp(**F**) dimer | 94.45 | 81.19 | 73.81 | 67.68 | 77.05 | 61.50 |
| **Glycosylation shift**[b] | **-0.12** | **+8.85** | **-0.29** | **-0.16** | **-0.16** | **-0.63** |
| β -Manp(**B**) of monomer | 94.47 | 81.24 | 73.81 | 67.74 | 77.11 | 61.53 |
| **Glycosylation shift**[b] | **-0.1** | **+8.9** | **-.29** | **-0.1** | **-0.1** | **-0.6** |
| NON-REDUCING END GROUP: | | | | | | |
| β -GlcpA(**C**) of dimer | 102.46* | 72.96 | 75.67 | 72.01 | 75.46 | n.r. |
| **Glycosylation shift**[c] | **+5.60** | **-1.99** | **-0.81** | **-0.70** | **-1.34** | **--** |
| β -GlcpA(**A**) of monomer | 102.46 | 73.05 | 75.68 | 72.01 | 75.2 | n.r. |
| **Glycosylation shift**[c] | **+5.6** | **-1.9** | **-0.8** | **-0.7** | **-1.6** | **--** |

A downfield shift relative to the model compound is considered positive
Chemical shift values relative to internal acetone (δ 31.0)
* Twin signal at 104.47 p.p.m. (see text)
a =model compound α -Manp-OH[98]
b = model compound β -Manp-OH
c = model compound β -GlcpA[94]

TABLE 23. $^{1}$H-N.M.R. DATA (200 MHz) OF MANNOSE END GROUP AND GlcpA END GROUP OF THE DIMER (14) FROM Hakea sericea GUM

| END GROUP : | CHEMICAL SHIFT (p.p.m.) H-1 | H-2 | H-3 | H-4 | H-5 | H-6 |
|---|---|---|---|---|---|---|
| REDUCING END GROUP: | | | | | | |
| α - Manp(**F**) of dimer | 5.27 | 4.03 | 3.80 | 3.65 | 3.79 | 3.80 |
| **Glycosylation shift**[a] | **+0.09** | **+0.17** | **+0.01** | **-0.01** | **-0.03** | **--** |
| α -Manp(**B**) of monomer | 5.27 | 4.06 | 3.86 | 3.65 | 3.79 | 3.80 |
| **Glycosylation shift**[a] | **+0.09** | **+0.14** | **+0.01** | **-0.01** | **-0.03** | **--** |
| β -Manp(**F**) of dimer | 4.96 | 4.12 | 3.56 | 3.64 | 3.38 | n.r. |
| **Glycosylation shift**[b] | **+0.06** | **+0.18** | **-0.26** | **+0.08** | **+0.07** | **--** |
| β -Manp(**B**) of monomer | 4.96 | 4.12 | 3.48 | 3.65 | 3.41 | n.r. |
| **Glycosylation shift**[b] | **+0.06** | **+0.18** | **-0.18** | **+0.07** | **+0.04** | **n.r.** |
| NON-REDUCING END GROUP: | | | | | | |
| β -GlcpA(**C**) of dimer | 4.55 | 3.38 | 3.55 | 3.56 | n.r. | -- |
| **Glycosylation shift**[c] | **-0.19** | **-0.07** | **-0.13** | **-0.09** | **--** | **--** |
| β -GlcpA(**A**) of monomer | 4.56 | 3.38 | 3.54 | 3.58 | 4.00 | -- |
| **Glycosylation shift**[c] | **-0.20** | **-0.07** | **-0.12** | **-0.11** | **-0.16** | **--** |

A downfield shift relative to the model compound is considered positive
Chemical shift values relative to internal acetone ( δ 2.23)
Glycosylation shifts were measured relative to model compounds:
a α -Manp-OH
b β -Manp-OH
c β -GlcpA

**F, B,C and A** Structures shown in Table 22

3.7.6.1.1.1 **In-chain mannose and glucuronic acid in dimer (14)**

By comparison with the proton resonances obtained for the C. speciosa dimer[17], the signal at 5.42 p.p.m. (1 H, singlet) was attributed to H-1 of the in-chain Man. This signal was then used in the COSY experiment to assign H-2 of the mannose. In the $^{13}C$ spectrum, the two C-6 signals of the mannose residues were assigned by the use of the APT experiment and use of the 3:1 ratio for $\alpha$ : $\beta$ anomers, thus leaving only one signal in this region of the spectrum to be identified viz. the in-chain C-6. The connectivities of the latter carbons with their appropriate H-6's and the aforementioned H-1 and H-2 protons of Man with their appropriate carbons could then be assigned unambiguously by the HETCOR experiment.

The H-1 of in-chain GlcpA of the dimer of C. speciosa resonates at 4.49 p.p.m. However in this region of the spectrum of **14**, there are only three peaks instead of the expected four for the 2 doublets of the in-chain and end-group glucuronic acid residues[17]. These three peaks must have originated from the partial overlap of the 2 doublets of the GlcpA anomeric signals. Since one of the doublets has already been assigned, the other must be due to the in-chain GlcpA unit. This overlap allows only an approximation of

chemical shifts, coupling constants and integral proton values for the anomeric protons of the GlcpA units of the dimer, which are mentioned in the following discussion.
The doublet at δ 4.49 attributed to H-1 of the in-chain GlcpA of **14** provided a starting point for the assignment of H-2 and H-3 by the COSY experiment[17].

In addition to the doublet at δ 4.49 (0.8H,$J_{1,2}$ ca. 8.5 Hz), another doublet at δ 4.68 (0.2H, $J_{1,2}$ ca. 7.7 Hz ) was also assigned to H-1 of in-chain β -GlcpA. The former was due to the α-anomer and the latter the β-anomer of the dimer respectively. This is indicative of the phenomenon of twinning[99,100] which is attributed to the different stereoelectronic environments which the substituted in-chain GlcpA experiences from the anomeric centre of the 2-linked mannose, resulting in a relatively large shift difference of 0.18 p.p.m. This environment could also influence conformational properties of the dimer, which in turn could be responsible for the increase in the chemical shift difference, above the value of 0.11 p.p.m. shown for the monomer[94].

All these previously assigned proton resonances of Man and GlcpA were used to establish connectivities with their appropriate $^{13}C$ atoms. The $^{13}C$ resonance assignments were reaffirmed when the remaining signals (of these two in-chain units) were assigned by comparison with model compounds men-

tioned earlier, ranging from monosaccharides to the polymer. The trimer (OTBT) and polymer (OTB) of Ornithogalum thyrsoides were subjected to $^{13}C$-n.m.r. and gave spectra containing 21 and 12 major resonances respectively[37], (Figures 17 and 18).

The 12 signals of the latter spectrum probably originated from the in-chain Man and GlcpA residues since in a molecule of this size the resonances of the two end-group units would be of negligible intensities when compared with those of the in-chain units. Also as the value of n increases from OTBT to OTB, those resonances of the latter which are also present in OTBT grow in intensity at the expense of the other $^{13}C$ signals also present in OTBT. Thus comparison of the in-chain units of the dimer with these model compounds, especially OTB (Tables 24 and 25), permitted relatively accurate assignments for the $^{13}C$ atoms which in turn allowed the connectivities with their protons to be established by the HETCOR experiment (Table 26).

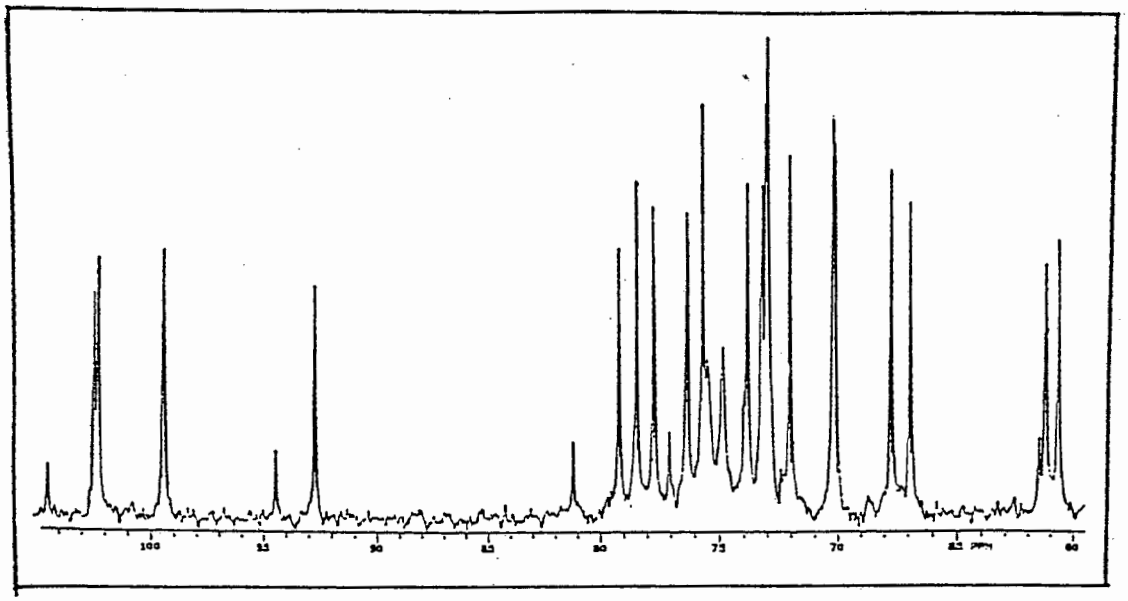


FIGURE 16 $^{13}C$-N.M.R. SPECTRUM (50.3 MHz) OF 14

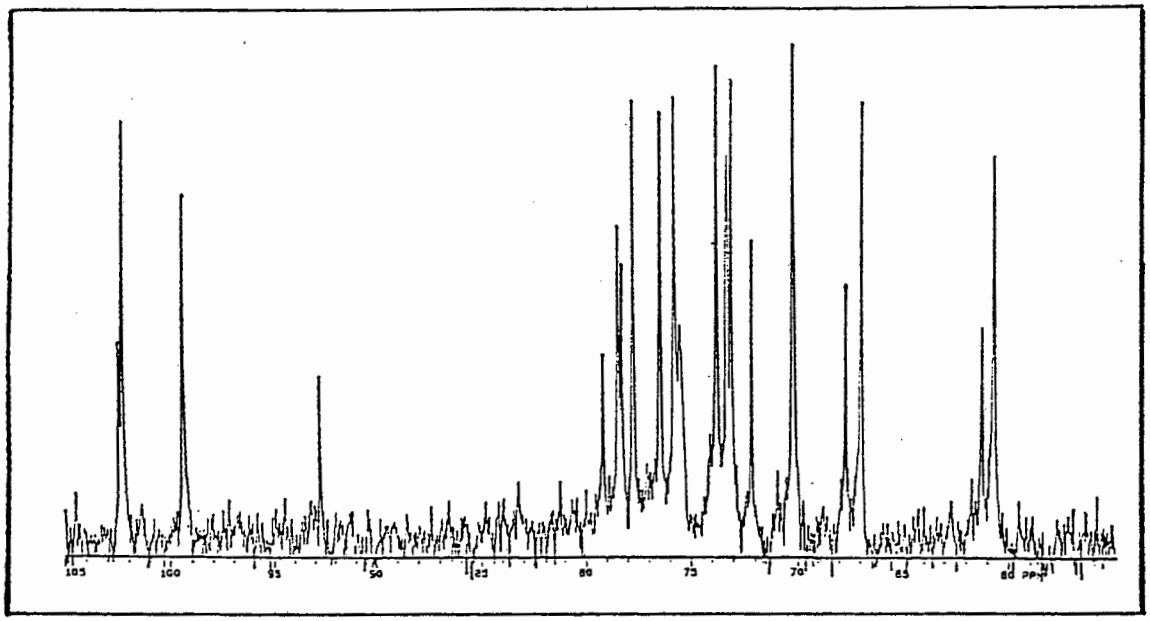


FIGURE 17. $^{13}C$-N.M.R. SPECTRUM (50.3 MHz) OF THE TRIMER FROM Ornithogalum thyrsoides[37](OTBT)

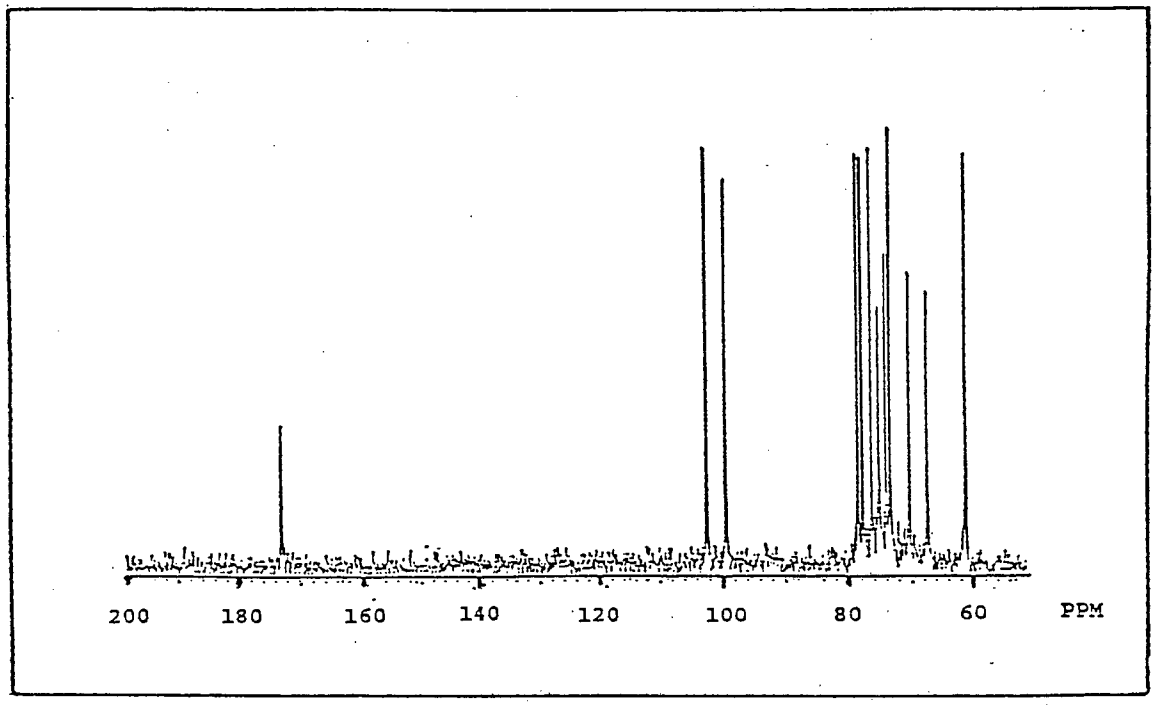



FIGURE 18. $^{13}C$-N.M.R.(50.3 MHz) SPECTRUM OF THE POLYMER FROM Ornithogalum thyrsoides[37](OTB)

TABLE 24. $^{13}C$-N.M.R. DATA (50.3 MHz) OF THE IN-CHAIN MANNOSE (D)[a] OF THE DIMER (**14**) FROM Hakea sericea GUM AS COMPARED WITH VARIOUS MODEL COMPOUNDS.

THE FORMULA OF THE DIMER (**14**) IS

β -D-GlcA-(1-2)- α -D-Man-(1-4)- β -D-GlcA-(1-2)-α, β -D-Man-OH
**C** **D** **E** **F**

| | DIMER (**14**) | FREE MANNOSE | MONOMER (**3**) | REDUCING END GROUP Man-OH (F) OF DIMER | OTBT (n=3) | OTB (n>10) |
|---|---|---|---|---|---|---|
| C - 1 | 99.43[b] **(+4.46)** | 95.5 | 92.77 | 92.72 | 99.3 | 99.94 |
| C - 2 | 78.43[c] **(+6.74)** | 72.3 | 79.39 | 79.18 | 78.43 | 78.8 |
| C - 3 | 70.11[d] **(-1.24)** | 71.9 | 70.15 | 70.18 | 70.22 | 70.5 |
| C - 4 | 66.89[e] **(-1.15)** | 68.5 | 67.74 | 67.68 | 66.97 | 67.6 |
| C - 5 | 72.96 **(-0.40)** | 73.9 | 73.06 | 73.22 | 72.80 | 73.9 |
| C - 6 | 60.63 **(-1.50)** | 62.6 | 61.23 | 61.2 | 60.69 | 61.3 |

Glycosylation shifts given in parentheses
A downfield shift relative to the model compound is considered positive
a Unit as designated in Table 22
b Model compound is dimer of C. speciosa[17]
c and d = COSY experiment used in assignment
e Model compounds are OTBT[37,101] and monomer, **3**
OTBT and OTB = the trimer and polymer of GlcA-2Man, isolated from Ornithogalum thyrsoides

TABLE 25. COMPARISON OF THE $^{13}C$-N.M.R. DATA (50.3 MHz) OF IN- CHAIN GlcpA (**E**)[a] OF THE DIMER (**14**) FROM Hakea sericea GUM WITH VARIOUS MODEL COMPOUNDS.

THE FORMULA OF THE DIMER (**14**) IS
β -D-GlcA-(1-2)- α -D-Man-(1-4)- β -D-GlcA-(1-2)-α, β -D-Man-OH
**C** **D** **E** **F**

| | DIMER (**14**) | FREE $Glc_pA$ | MONOMER (**3**) | GlcpA (**C**) OF DIMER | OTBT[b] | OTB[c] |
|---|---|---|---|---|---|---|
| C - 1 | 102.26[d] **(+5.45)** | 96.81 | 102.46 | 102.48 | 102.45 | 102.43 |
| C - 2 | 73.97[e] **(-0.95)** | 74.92 | 73.05 | 72.96 | 73.07 | 73.3 |
| C - 3 | 76.26[f] **(-0.17)** | 76.46 | 75.68 | 75.67 | 76.43 | 76.6 |
| C - 4 | 77.71[g] **(+4.99)** | 72.01 | 72.01 | 72.02 | 77.71 | 78.0 |
| C - 5 | 74.84[h] **(-1.99)** | 76.83 | 75.24 | 75.46 | 75.49 | 75.1 |
| C - 6 | n.r. | 176.75 | n.r. | n.r. | n.r. | 175.4 |

A downfield shift relative to the model compound is considered positive.
Chemical shift values relative to internal acetone (δ 2.23)
a Unit as designated in Table 22
b $(GlcA\text{-}2Man)_n$ where n=3 isolated from O. thyrsoides
c Polymer of GlcA-2Man isolated from O. thyrsoides
d Model compound dimer of C. speciosa[17]
e COSY experiment used in assignment
f,g and h Refer to values in reference 76
n.r. not resolved

These assigned $^{13}C$-n.m.r. signals allowed the assignment of the proton resonances using the HETCOR experiment, Table 26.

TABLE 26. $^{1}$H-N.M.R. DATA (200 MHz) OF IN-CHAIN MANNOSE (**D**)[a] AND GlcpA (**E**)[b] OF THE DIMER (**14**) FROM Hakea sericea GUM.

THE FORMULA OF THE DIMER (**14**) IS
β -D-GlcA-(1-2)- α -D-Man-(1-4)- β -D-GlcA-(1-2)- α, β -D-Man-OH
**C** **D** **E** **F**

| | CHEMICAL SHIFT (p.p.m.) | | | | | |
|---|---|---|---|---|---|---|
| IN-CHAIN GROUP | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 |
| MANNOSE[a] | 5.42 | 4.17 | 3.95 | 3.73 | 3.37 | 3.78 |
| | **(+0.24)** | **(+0.31)** | **(+0.1)** | **(+0.07)** | **(-0.45)** | **--** |
| GlcpA[b] | 4.49* | 3.36 | 3.59 | 3.94 | n.r. | -- |
| | **(-0.25)** | **(-0.16)** | **(-0.01)** | **(+0.17)** | **--** | **--** |

Glycosylation shifts given in parentheses.
A downfield shift relative to the model compound is considered positive.
* Twin signal at δ 4.68
a and b Units as designated in Table 22
Glycosylation shifts were measured relative to model compounds:
a α -ManpOH
b β -GlcpA

All the assignments for the carbons and protons are presented in Table 27.

TABLE 27. SUMMARY OF CHEMICAL SHIFTS AND GLYCOSYLATION SHIFTS OF THE CARBONS AND PROTONS OF THE DIMER (14) FROM Hakea sericea.

THE FORMULA OF THE DIMER (14) IS

β -D-GlcA-(1-2)- α -D-Man-(1-4)- β -D-GlcA-(1-2)-α, β -D-Man-OH
C D E F

| | CHEMICAL SHIFT (p.p.m.) | | | | | |
|---|---|---|---|---|---|---|
| | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| α-Man$_p$ -OH (F) | 92.72 (-2.25) | 79.18 (+7.49) | 70.18 (-1.17) | 67.68 (-0.36) | 73.22 (-0.14) | 61.20 (-0.93) |
| β-Man$_p$ -OH (F) | 94.45 (-0.12) | 81.19 (+8.85) | 73.81 (-0.29) | 67.68 (-0.16) | 77.05 (-0.16) | 61.50 (-0.63) |
| in-chain Man (D) | 99.43 (+4.46) | 78.43 (+6.74) | 70.11 (-1.24) | 66.89 (-1.15) | 72.96 (-0.40) | 60.63 (-1.50) |
| β-GlcpA (C) | 102.46* (+5.60) | 72.96 (-1.99) | 75.67 (-0.81) | 72.01 (-0.70) | 75.46 (-1.34) | n.r. -- |
| in-chain GlcpA (E) | 102.26 (+5.45) | 73.97 (-0.95) | 76.26 (+0.17) | 77.71 (+4.99) | 74.84 (-1.99) | n.r. -- |

| | CHEMICAL SHIFT (p.p.m.) | | | | | |
|---|---|---|---|---|---|---|
| | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 |
| α-Manp -OH (F) | 5.27 (+0.09) | 4.03 (+0.17) | 3.80 (+0.01) | 3.65 (-0.01) | 3.79 (-0.03) | 3.80 -- |
| β-Manp -OH (F) | 4.96 (+0.06) | 4.12 (+0.18) | 3.56 (-0.26) | 3.64 (+0.08) | 3.38 (+0.07) | n.r. -- |
| in-chain Man (D) | 5.42 (+0.24) | 4.17 (+0.31) | 3.95 (+0.1) | 3.73 (+0.07) | 3.37 (-0.45) | 3.78 -- |
| β-GlcpA (C) | 4.55 (-0.19) | 3.38 (-0.07) | 3.55 (-0.13) | 3.56 (-0.09) | n.r. -- | -- -- |
| in-chain GlcpA (E) | 4.49 (-0.25) | 3.36 (-0.16) | 3.59 (-0.01) | 3.94 (+0.17) | 4.02 (-0.14) | -- -- |

A downfield shift relative to the model compound is considered positive
Chemical shift values relative to internal acetone ( δ 2.23)
Glycosylation shifts were measured relative to model compounds given previously.

### 3.7.6.1.2 **F.a.b.-m.s. of permethylated product of 14**

#### 3.7.6.1.2.1 **Positive f.a.b.-m.s of 14**

The spectrum obtained on positive f.a.b.-m.s. of permethylated **14** (Fig. 19) showed the presence of a number of fragment ions.

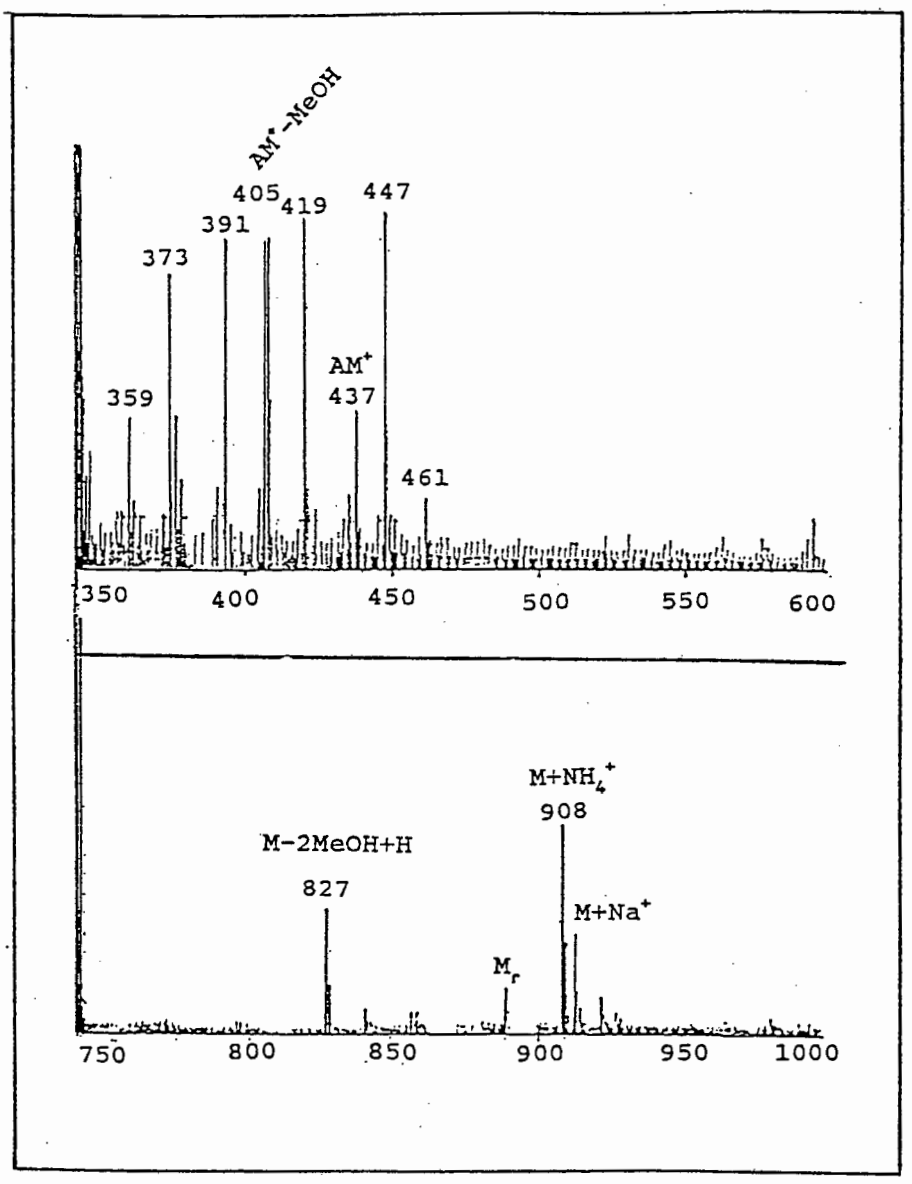



FIGURE 19. F.A.B.-M.S. (POSITIVE MODE) OF DIMER (**14**) FROM H. sericea.

The peaks at m/z values 913 and 908 are attributed to the cationised molecular ions $[M + Na]^+$ and $[M + NH_4]^+$ respectively. Other fragment ions, which could be assigned, are listed below

| m/z value | Permethylated component |
|---|---|
| 405 | $AM^+$ - MeOH |
| 437 | $AM^+$ |
| 827 | M - 2 MeOH + H |
| 890 | $M_r$ |

The molecular ion, $M_r$ = 908 - 18 (ie. $H_2O$)

These fragment ions indicate probable glycosidic cleavage of the Pathway A (also termed type $A_1$) where the charge is retained on the non-reducing end as the oxonium ion[79] (Fig 20). Some general trends in fragmentation like those present in larger oligomers[31,37] were not obviously present in the monomer and the dimer.

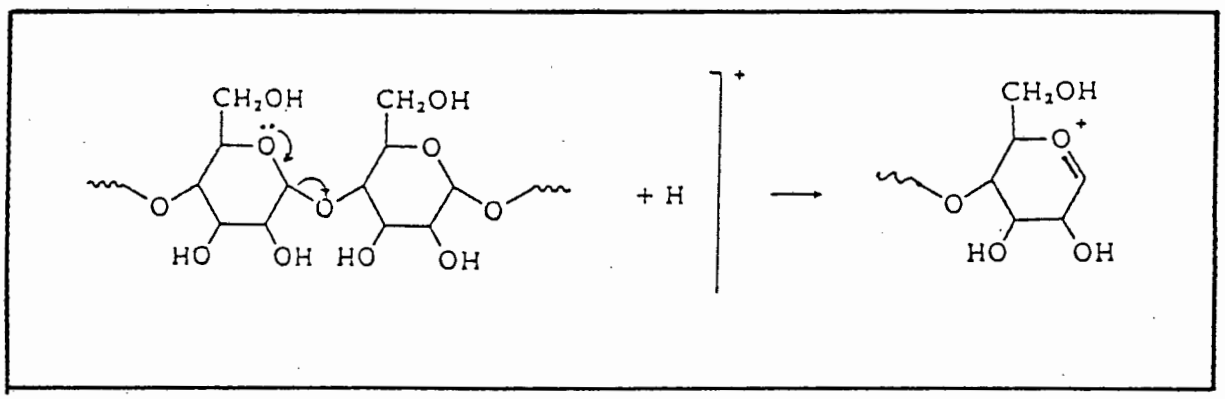



FIGURE 20. PATHWAY A MECHANISM

The possibility of Pathway D[79] fragmentation of this fraction is hinted at by the presence of m/z ions 447, 419 and 391 which show differences of 28 mass units. These ions could have resulted from ring cleavage with the charge being retained on the reducing end. The resultant ions 419 and 447 are 28 mass units heavier than those in Pathway A.

### 3.7.6.1.2.2 **Negative f.a.b.-m.s. of 14**

The spectrum obtained from negative f.a.b.m.s. of the native dimer (Fig. 21) showed a major peak of m/z 693 corresponding to the $[M-H]^-$ ion; the molecular ion is composed of two GlcA units each with m/z 176 and two Man units each with m/z of 162 plus 18 ($H_2O$). Evidence for the monomer, present as a

result of ionisation of the dimer, can be seen by the peak at m/z 355; similarly the GlcA-Man-GlcA sequence is indicated by the peak at m/z 531 (176 + 162 + 176 + 18). The phenomenon of ionization dimer formation[79] is exemplified by the $[2M-H]^-$ peak at 1388, (ie. 2 X 694), which is within the limit of ± 1 noted for negative f.a.b.-m.s. of native oligosaccharides.

The sequence of ionisation is presented below:

$$1388 \xrightarrow{(-694)} 694\ (693) \xrightarrow{(-162)} (531) \xrightarrow{(-176)} (355)$$

( ) indicates the components which are "related" in a sequence

The value of the f.a.b.-m.s. technique for the accurate assessment of molecular weight and indication of sequencing of the sugar components of oligosaccharides is clear in this application, especially since sequencing is not often seen in f.a.b.-m.s. of underivatised oligomers[79]. The method is thus an invaluable tool in polysaccharide studies, especially where degradation products are examined.

The fragmentation patterns are simple in this study of the dimer, with relatively few peaks in the spectrum in contrast with underivatised carbohydrates where patterns B and C are indistinguishable and often complicated by "double cleavages" which make sequencing difficult[79].

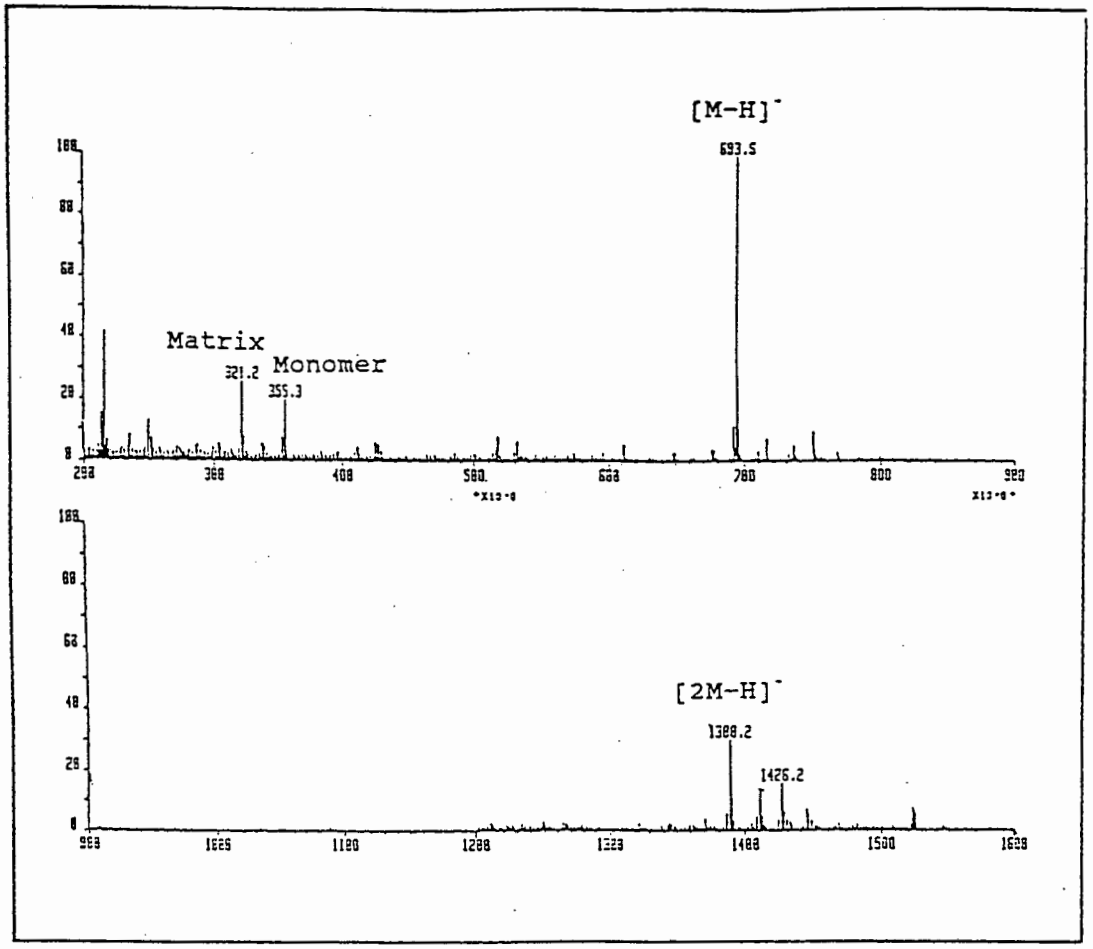



FIGURE 21. F.A.B.-M.S. (NEGATIVE MODE) OF THE DIMER (**14**) FROM Hakea sericea.

# 4. INVESTIGATION OF THE POLYSACCHARIDE EXUDATE FROM Hakea gibbosa (HG). (i) DISCUSSION

## 4.1 INTRODUCTION

Hakea gibbosa (rock hakea) is indigenous to New South Wales, Australia, where the plants are referred to as "needlebushes", and also occurs in the sandstone areas around Sydney[46]. In 1830 it was brought into the Republic of South Africa and planted in the Cape area to serve as a ready source of firewood because of its prolific growth characteristics. H. gibbosa grows in the Cape Peninsula, in the area between Caledon and Hermanus in the Cape Province and to a lesser extent in the Franschhoek mountains. Its prolific growth and density of growth are a threat to the natural fynbos vegetation of the region. The extremely hard fruit, like that of H. sericea, is unaffected by fire and even after burning the seeds are distributed widely by the wind. These plants carry seeds even when they are only a few centimetres high.

H. gibbosa is different from H. sericea in that the plants grow to a height of approx. 4 metres, with thicker and longer leaves of 40 to 80 mm length and 1.5 to 2.0 mm in diameter; its fruit is larger and the flowers a deeper cream colour.

The sample of gum was collected from the bark of trees growing on the Constantiaberg near Constantia Nek, W.Cape. The trees were classified as H. gibbosa (Sm) Cav. by Dr John Rourke of Kirstenbosch Botanical Gardens. A photograph of H. gibbosa trees appears below.

The results obtained from the various experimental aproaches applied to Hakea gibbosa (HG) gum presented in this chapter are compared with those already reported (Chapter 3) for H. sericea gum (HSWS), examined by similar analytical procedures.

## 4.2 ORIGIN AND ISOLATION OF THE POLYSACCHARIDE FROM Hakea gibbosa GUM

The freshly exuded gum was totally soluble in water at room temperature, in contrast to the dry gum from the bark of H. sericea, which dissolved easily only on heating. On characterisation (Table 28; Fig. 23.a) the proportion of protein in the polysaccharide was shown to be insignificant; the major sugar components were Ara, Xyl, Man, Gal, GlcA in the ratio 6:1.5:1:8:2.5. The Man content was lower than that of GlcA, differing in this respect from HSWS in which where they were equal. However this difference may be due in part to the resistance of the glycosyl bond between GlcA and Man to acid hydrolysis, which could be strengthened by the location of these residues in the innermost core of the structure, where they could be among the last of the residues to be exposed to TFA. Thus, less Man would be detected after 18 h of exposure to TFA. Longer exposure would not necessarily increase the amount of Man detected since there is likely to be more destruction of the Man residues by TFA[22,88].

## 4.3 METHYLATION OF *Hakea gibbosa* GUM

The procedure adopted in methylation, including the monitoring of the degree of methylation and the characterisation of the GlcA linkages by reduction with LAD, was essentially the same as that used for HSWS (see section 3.3). After purification the methylated product (MHG) was hydrolysed and the derived alditol acetates were subjected to g.l.c. and g.l.c.-ms analysis (Table 29 and Fig. 22).

The main conclusions are given below :

Arabinose: The bulk of the arabinose was terminal and in the furanose form, but the presence of some 2,3-Ara indicated the occurrence of (1-4)-linked Arap or (1-5)-linked Araf (*ca*. 20 % of total Ara). The 2,5-Ara detected in the methylation analysis of HSWS was not found here; qualitatively the results were otherwise similar to those given by HSWS.

Xylose: All Xyl was present as end-group in the pyranosyl form.

Mannose: The Man was present only as 2,3-linked units, in contrast to that of MHSWS, where a trace amount of 2,3,6-linked Man was detected.

TABLE 28. PROPERTIES OF GUM FROM Hakea gibbosa

| PROPERTIES | |
|---|---|
| Water solubility | Totally soluble[a] |
| $[\alpha]_D$ | -8° (c 1.0) |
| Micro analysis: | |
| C (%) | 43.5 |
| H (%) | 6.0 |
| N (%) | < 0.5 |
| Sugar proportions[b](mol %) | |
| Ara | 32 |
| Xyl | 8 |
| Man | 5 |
| Gal | 43 |
| GlcpA[c] | 12 |
| $\bar{M}_w$[d] | $>2.10^6$ |
| Acid equivalent | 1400 |

a Soluble at a concentration of 2% in water at room temperature

b Proportions of neutral sugars determined as alditol acetates, on column A

c Determined by the colorimetric method of Blumenkrantz et al.[85]; quoted as anhydride form

d Single peak on Sepharose 4B

TABLE 29. METHYLATION ANALYSIS DATA FOR *H. gibbosa* (HG) GUM AND LAD-REDUCED METHYLATED HG

| PARTIALLY METHYLATED ALDITOL ACETATES[a] | LINKAGE MODES | METHYLATED HG (mol %) (corrected for GlcA) | LAD-REDUCED METHYLATED (mol %) |
|---|---|---|---|
| 2,3,5-Ara | T-Araf | 21 | 18 |
| 2,3-Ara | - 4)-Arap or - 5)-Araf | 5 | 2 |
| 2,3,4-Xyl | T-Xylp | 9 | 8 |
| 2,3,4,6-Gal | T-Gal | 12 | 13 |
| 2,4,6-Gal | - 3)-Galp | 4 | 3 |
| 2,3,6-Gal | - 4)-Galp | 2 | 3 |
| 2,3,4-Gal | - 6)-Galp | 12 | 22 |
| 2,4-Gal | - 3,6)-Galp | 8 | 10 |
| 2-Gal | - 3,4,6)-Galp | 2 | 5 |
| 4(3)-Gal | - 2,3,6)-Galp or - 2,4,6)-Galp | 7 | 9 |
| 4,6-Man | - 2,3)-Manp | 6 | 3 |
| 2,3-Glc($d_2$)[b] | - 4)-GlcpA | - | tr |
| 2-Glc($d_2$) | - 3,4)-GlcpA | - | 3 |

a 2,3,4,6-Gal = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol, etc

b Determined after LAD-reduction of the permethylated product

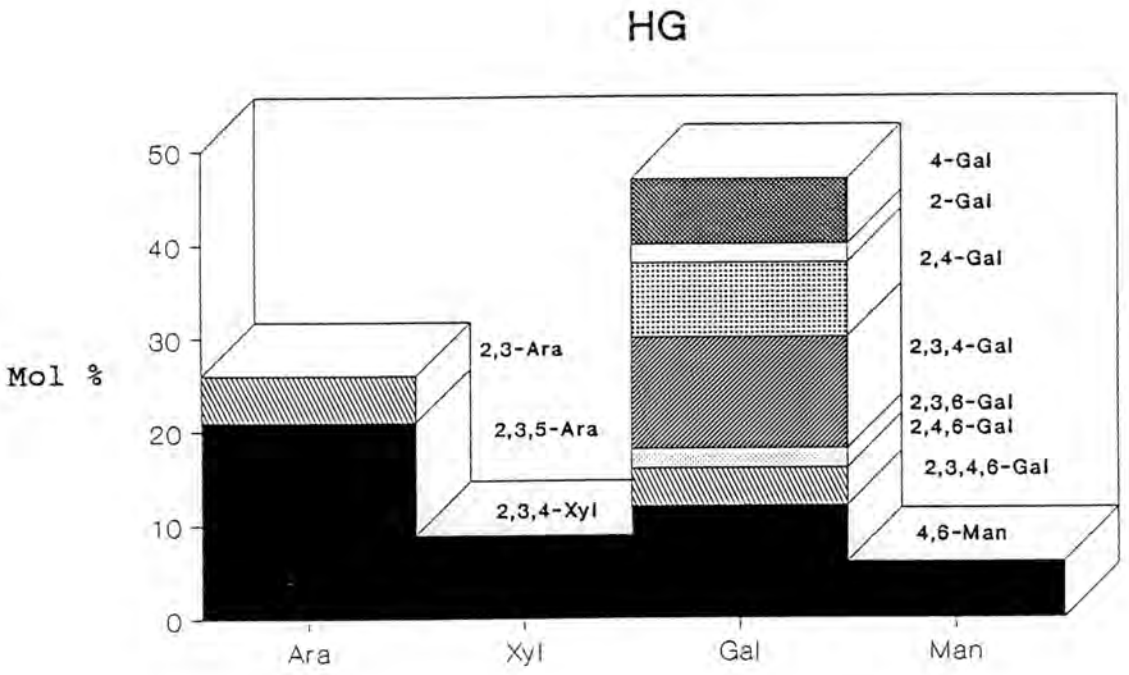



FIGURE 22. HISTOGRAM OF METHYL ETHERS IN METHYLATED H. gibbosa GUM

Glucuronic acid: LAD reduction revealed the presence of GlcA residues of two types viz. the 3,4-linked and the 4-linked forms with the latter present in trace amounts only. From the results of the analysis of LAD-reduced MHG the ratio of GlcA to Man was found to be approximately 1:1, albeit that the actual amounts detected were low (<3%). This result again infers a close relationship between these two residues in the polysaccharide structure as was noted in H. sericea gum.

Galactose: The units identified were all of the pyranosyl form and had the following proportions and types of linkages:

As in MHSWS the major two components present, in equal amounts, were 6-linked and T-Gal. In MHG 3,6-linked and 2,3,6- or 2,4,6-linked units were present in the ratio of 1:1. The proportion of the 3-linked Gal was half that in MHSWS; the proportions of 4-linked Gal were equal in the two gums. Generally all the methyl ethers detected in MHG were also present in MHSWS, but some extra components detected in MHSWS in trace amounts, viz those corresponding to 4,6-, 3,4-, 2,6- and 2,4-linked galactosyl units, were not found here.

The wide range of linkages found among the five component sugars, viz. Gal, Ara, Xyl, Man and GlcA, strongly suggests that this high molecular weight polysaccharide HG is, like HSWS, a highly branched structure composed of a large galactan network located around a core structure composed of GlcA, Man and Xyl.

## 4.4 SMITH DEGRADATION OF *Hakea gibbosa*

### 4.4.1 First Smith Degradation of HG (Scheme 9)

The results of methylation analysis indicate that periodate oxidation of HG could cleave 61% of the constituent residues, yielding a periodate-resistant product that would be a valuable source of structural information. A large sample of HG (30 g) was subjected to Smith degradation to allow isolation in amounts sufficient for characterisation. The periodate consumption of 6.2 mmol $g^{-1}$ showed over-oxidation to an extent of 5%, which was not considered excessive.

Two products were obtained on fractionation with a methanol-acetone mixture, a high molecular-weight insoluble product (SD1; Table 30; 38% w/w) which differed from HS-SD1 in that it contained no Ara and Xyl, and a soluble fraction (SD1 solubles; 51%) composed of glycerol (8+), Ara (+), Gal (+) and a trace of Xyl.

The SD1 product was shown on elution from Sepharose 4B to have $\bar{M}_w$ 300 000 (Fig. 23) which was 25% larger than that of the SD1 product of *H. sericea* gum (Fig. 3.b). However, as in HS-SD1 this $\bar{M}_w$ indicated some cleavage at periodate-vulnerable points within the polysaccharide structure, in addition to removal of peripheral sugars, *viz*. arabinose,

xylose and galactose. This could imply the presence of periodate-vulnerable sugar(s) joining adjacent periodate-resistant units each with $\bar{M}_w$ 300 000.

TABLE 30. PROPERTIES OF SMITH DEGRADATION PRODUCTS FROM HG

| PROPERTIES | HG-SD1 | HG-SD2 |
|---|---|---|
| $[\alpha]_D$ (c 1.0) | +55° | + 64° |
| Periodate consumption (mmol $g^{-1}$) | 6.1 | n.d. |
| Molecular weight distribution | 320 000 and 280 000 | 6000 and 2660 |
| $\bar{M}_w$ | 300 000 | 4 400 |
| Glycolaldehyde[a] (%) | - | 1.0 |
| Acid equivalent | 1 000 | 630 |
| Sugar composition (mol %) | | |
| Man | 24 | 29 |
| Gal | 56 | 44 |
| GlcA[b] | 20 | 27 |
| Methylation analysis (mol %) | | |
| 2,3,4,6-Man | tr | tr |
| 4,6-Man | 8 | 25 |
| 2,3,4,6-Gal | 16 | 30 |
| 2,4,6-Gal | 29 | 21 |
| 2,3,4-Gal | 17 | - |
| 2,4-Gal | 2 | 3 |
| 4(3)-Gal | 4 | tr |
| 2-Glc($d_2$)[c] | 10 | tr |
| 2,3-Glc($d_2$) | 9 | 21 |

a Determined by colorimetric method of Dische[93]
b Determined by colorimetric method of Blumenkrantz[85]
c Determined by LAD reduction[91]

Analysis of component sugars in the SD1 product indicated total removal of all the Ara and Xyl from HG, with localisation or concentration of GlcA and Man in ratio ca. 1:1 (identical to that in HS-SD1). Since there are only three sugar components in the SD1 product from HG a close relationship between the GlcA and Man seems more probable in this case than it did in HSWS where the first Smith degradation produced only a drop in concentration of Ara, Xyl and Gal.

Methylation data for SD1 showed that the preponderant residues were T-Gal, 3-linked Gal and 6-linked Gal, in a ratio 1:2:1. As with MHSWS the diminished proportion of 2,4-Gal suggested that the linkage of periodate-vulnerable substituents such as T-Araf, Xylp or T-Galp was to the O-6 of the otherwise 3-linked Galp or to the O-3 of the otherwise 6-linked Gal. Reasons for the increases in 6-linked Galp and 3-linked Galp are as mentioned before for HS-SD1 (section 3.4.1.1). All the Man was shown to be in the 2,3-linked form while the GlcA residues were found to be present in the 3,4- and 4-linked forms in ratio 1:1, which was different from that in HS-SD1 where the GlcA was mostly of the 3,4-linked form. The absence of Xyl taken in conjunction with the increased amount of 4-linked GlcA in HG-SD1 suggests close involvement of Xyl in the polysaccharide structure, some of the Xyl residues being linked to GlcA through its O-3 position.

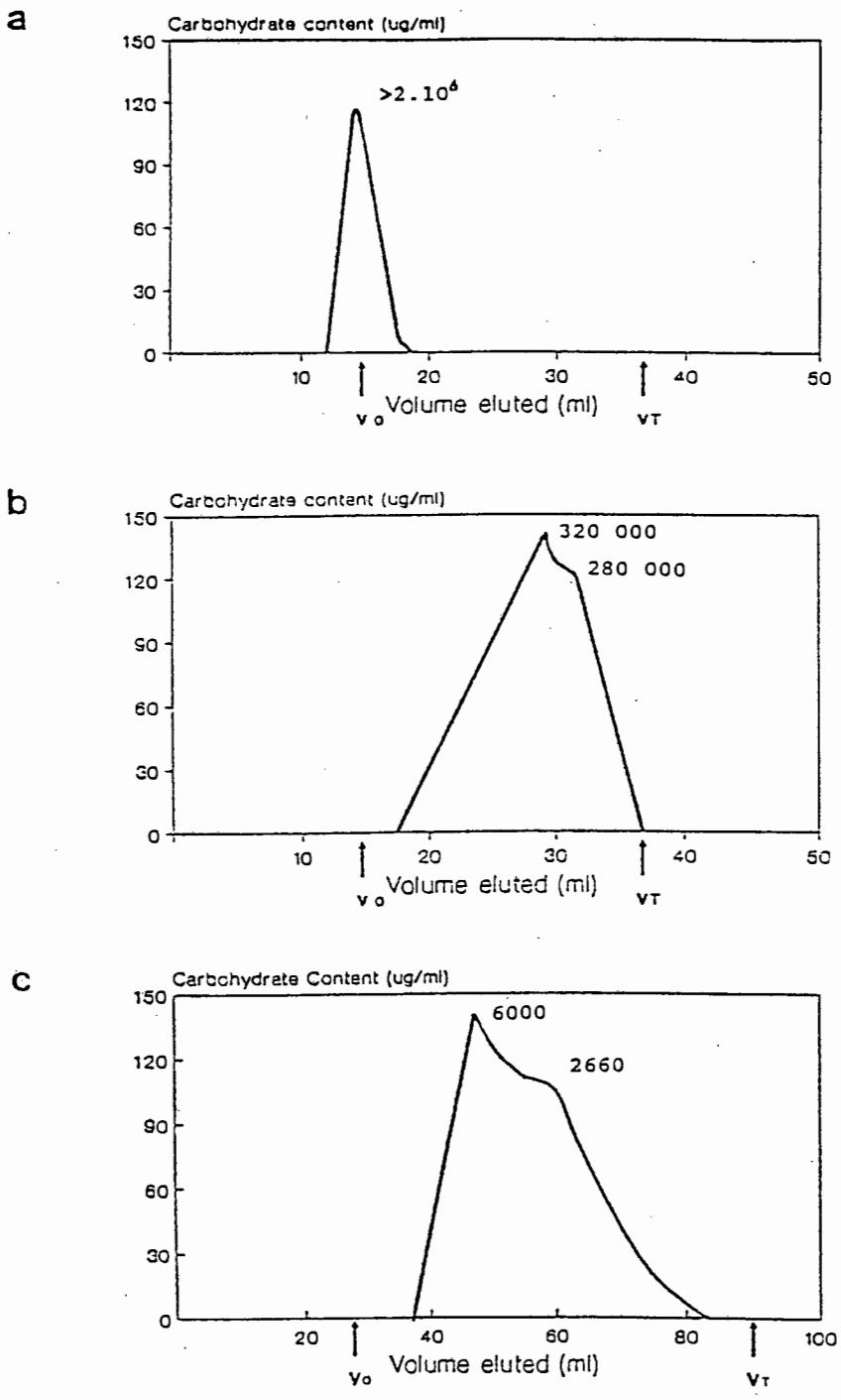



FIGURE 23. STERIC-EXCLUSION CHROMATOGRAPHY OF a. HG (SEPHAROSE 4B), b. HG-SD1 (SEPHAROSE 4B), AND c. HG-SD2 (BIO-GEL P-10)

The proportion of the methyl ether of Man detected was lower than expected from the neutral sugar composition,but this could be due in part, as mentioned, to the resistance of the glycosyl bond between GlcA and Man to acid hydrolysis.
The information obtained in this investigation and in that of HS-SD1 strongly suggests the structural possibility stated in section 3.4.1.1, viz. the involvement in the polysaccharide core of -4GlcA-2Man aldobiouronic acid units in which GlcA and Man are both substituted at their O-3 positions by Gal and/or xylose.

This core structure could also have a large envelope around it, composed mainly of Gal residues present as chains, terminating occasionally in Araf or Xylp but mainly in Galp.

### 4.4.2 Second Smith Degradation (Scheme 10)

The large proportion of periodate-vulnerable residues (43%) in HG-SD1 indicated the necessity for further Smith degradation. Oxidation of HG-SD1 (4 g) consumed 6.1 mmol $g^{-1}$ of periodate which almost equalled the theoretically calculated value of 6.12 mmol $g^{-1}$. The procedure outlined in Scheme 10 yielded a fraction insoluble in methanol-acetone mixture (25 % yield; HG-SD2) and another fraction (50% yield) soluble in the mixture. The soluble fraction was deionised in the usual manner and examination of the resulting syrup by p.c. revealed the presence of glycerol (10+) and a trace of Gal.

As suggested in section 3.4.1.1, the glycerol originated from the many periodate-vulnerable Galp residues in the HG-SD1. The HG-SD2 product was shown to contain 1% of glycolaldehyde which could be part of the acetals linking periodate-resistant blocks in the molecule or could exist as an attachment at the reducing end of HG-SD2. Since prolonged exposure to M TFA (40 days) removed this mole of glycolaldehyde without decreasing its $\overline{M}_w$ (4400), it must have been attached to the end of HG-SD2 and not in the interior of the molecule. Characterisation of HG-SD2 in the usual manner (Table 30) showed the presence of sugars Man, Gal and GlcA in a molar ratio ~ 3:4:3. Methylation analyses including LAD reduction of the carboxylate group of methylated SD2 revealed the following important structural information:

(i) The predominant components present were T-Galp, 3-linked Galp, 2,3-linked Man and 4-linked GlcA in ratio ~1.5:1:1:1. Quantitatively this was identical to that in the HS-SD2 product.

(ii) There is definite localization of the Man and GlcA as occurred in HS-SD2.

(iii) Components also present in HG-SD2, albeit in small amounts (each < 3%) were branch points of 3,6-linked Gal, and 2,3,6- or 2,4,6-linked Gal.

It was decided not to perform another Smith degradation on HG-SD2 but rather on an autohydrolysed fraction of HG.

## 4.5 PARTIAL HYDROLYSIS (AUTOHYDROLYSIS) OF HG

Autohydrolysis of HG was performed to remove most if not all of the peripheral residues present in the polysaccharide, viz. arabinofuranosyl, xylopyranosyl and some of the terminal galactopyranosyl groups. Treatment of HG with dilute sulphuric acid (Scheme 11) yielded a product, AHG, of $\bar{M}_w$ 880 000 (Fig. 24.a) that contained only a trace of Ara. The component sugars were Xyl, Man, GlcA and Gal in the ratio 1:1:1:4. (Table 31).

The proportions of GlcA and Man were approximately equal, as found in the case of autohydrolysed product from HSWS (i.e. A). Also the equimolar proportion of Xyl suggests involvement of this sugar in the core structure. The major components shown by methylation analysis (Table 32) were T-Gal, 3-linked Gal, 6-linked Gal, 2,3-linked Man and 4-linked GlcA in a ratio 3:1:3:1:1. Lesser amounts of 3,6-linked Gal and 3,4-linked GlcA (4% each) were also detected. The 4-linked and 3,4-linked forms of GlcA were present in ratio 1:0.4, which approximated to that in A.

TABLE 31. PROPERTIES OF AUTOHYDROLYSED HG (AHG)

| PROPERTY | AHG |
|---|---|
| $[\alpha]_D$ | + 45° |
| Water solubility | Totally soluble |
| Microanalysis: | |
| C (%) | 37.6 |
| H (%) | 5.3 |
| N (%) | 0.5 |
| Sugar proportions (mol %) | |
| Ara | tr |
| Xyl | 12 |
| Man | 14 |
| Gal | 56 |
| GlcA[a] | 18 |
| Acid equivalent | 975 |
| $\bar{M}_w$[b] | 880 000 |
| Yield (%) | 53 |

a Determined by the colorimetric method of Blumenkrantz[85]
b Determined on a column of Sepharose 4B

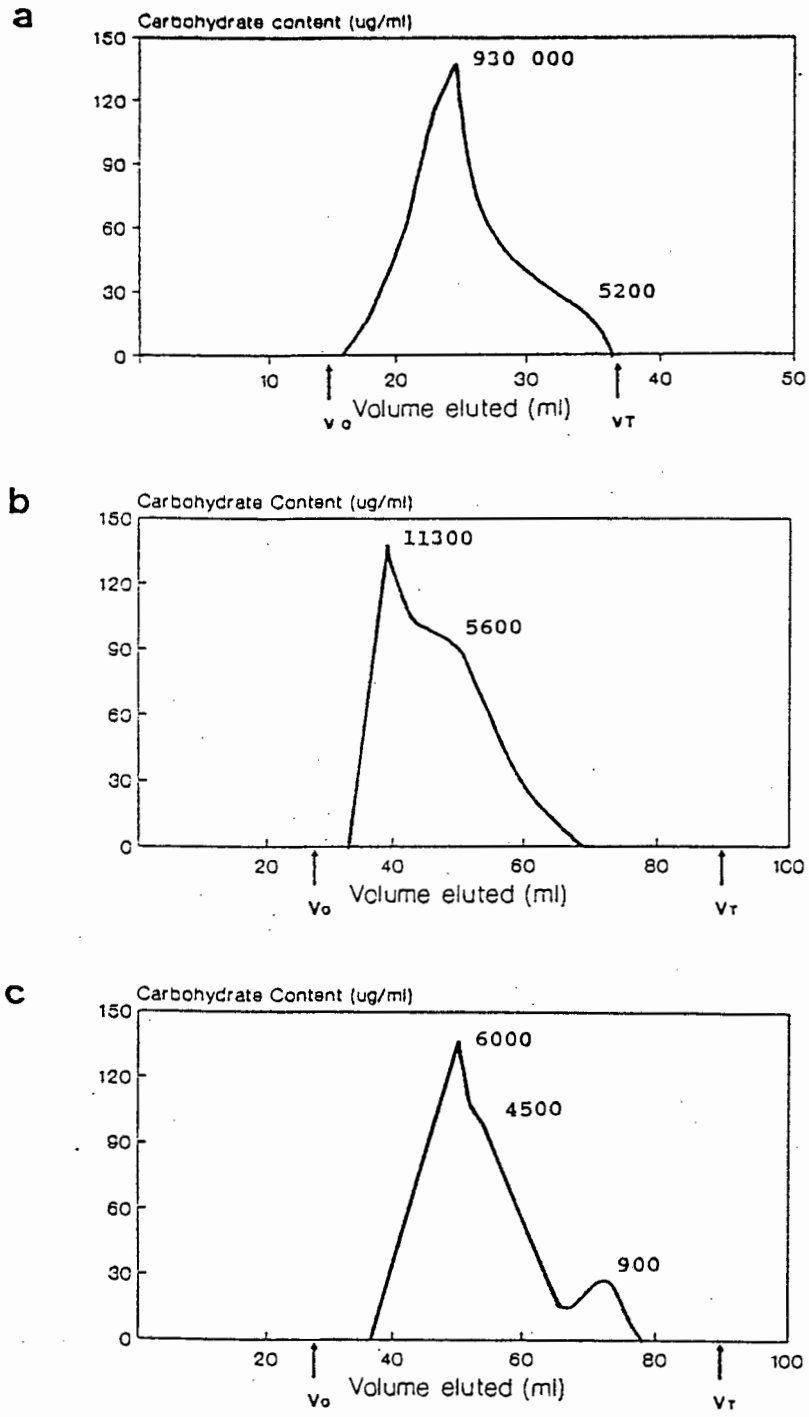



FIGURE 24. STERIC EXCLUSION CHROMATOGRAPHY OF a. AHG (SEPHAROSE 4B) b. AHG-SD1 (BIO-GEL P-10), and c. AHG-SD1-39 (BIO-GEL P-10)

TABLE 32. METHYLATION ANALYSIS OF AHG IDENTIFIED AS ALDITOL ACETATES

| PARTIALLY METHYLATED ALDITOL ACETATES | LINKAGE MODES | MOLAR PROPORTIONS (%) |
|---|---|---|
| 2,3,5-Ara | T-Arap | tr |
| 2,3,4-Xyl | T-Xylp | tr |
| 2,3,4,6-Man | T-Manp | tr |
| 2,3,4,6-Gal | T-Galp | 30 |
| 2,4,6-Man | -3)- Manp | tr |
| 2,4,6-Gal | -3)- Galp | 11 |
| 2,3,6-Gal | -4)- Galp | tr |
| 2,3,4-Gal | -6)- Galp | 29 |
| 4,6-Man | -2,3)- Manp | 11 |
| 2,4-Gal | -3,6)-Galp | 4 |
| 2,3-Gal | -4,6)-Galp | tr |
| 2-Gal | -3,4,6)-Galp | tr |
| 4(3)-Gal | -2,3,6)-Galp OR -2,4,6)-Galp | tr |
| 2,3,4-Glc ($d_2$)[a] | T-GlcA | tr |
| 2,3-Glc ($d_2$) | -4)-GlcA | 10 |
| 2-Glc ($d_2$) | -3,4)-GlcA | 4 |

a All Glc determined after LAD reduction of methylated AHG.

Because of the presence of a high proportion (69%) of residues which would be vulnerable to periodate AHG was subjected to Smith degradation.

### 4.5.1 Smith Degradation of Partially Hydrolysed HG (AHG; Scheme 12)

On Smith degradation two products were obtained, the insoluble (AHG-SD1; 28% yield) and the soluble fraction (66% yield); the latter contained Ara (tr), Xyl (+) and glycerol (10 +). The AHG-SD1 on characterisation (Table 33) was shown to be composed of Gal, Man and GlcA in a ratio of approximately 2.5:1:1.

TABLE 33. PROPERTIES OF SMITH DEGRADATION PRODUCTS FROM AHG

| PROPERTIES | AHG-SD1 | AHG-SD1-39 |
|---|---|---|
| $[\alpha]_D$ | +63° | +63° |
| Mol. wt. distribution | 11300 and 5600 (5:2.4) | 6000,4500 and 900 (7:4:1) |
| $\bar{M}_w$[a] | 9300 | 5000 |
| Acid equivalent | 808 | 730 |
| Glycolaldehyde (%) | 1 | - |
| Sugar composition (mol %) | | |
| Erythritol | tr | - |
| Glycerol | tr | - |
| Man | 21 | 21 |
| Gal | 56 | 57 |
| GlcpA[b] | 23 | 22 |

a Determined on a column of Bio-Gel P-10
b Determined by the colorimetric method of Blumenkrantz[85]

Also present in AHG-SD1 were traces of glycerol and erythritol, the latter probably originating, as in HS-SD3, from reduction of the lactone of the erythronic acid which originated from degraded GlcA. The acid equivalent of 808 suggests an acid content of ca. 24% which is accounted for by the GlcA (23%) and the small amount of erythronic acid. The glycolaldehyde content corresponded to a molar proportion of ca. 1 mol per mol AHG-SD1. Since prolonged exposure to M TFA for 39 days yielded a product (AHG-SD1-39), devoid of glycolaldehyde with a decreased $\bar{M}_w$ of 5000, it was assumed that the glycolaldehyde had been situated near the middle of the molecule.

The other properties of AHG-SD1-39 were not significantly different from those of the AHG-SD1 product (Table 33). The methylation data (Table 34) revealed the major components to be T-Gal, 3-linked Gal and 2,3-linked Man in a ratio ~1:1:1. Other compounds shown to be present in AHG-SD1, albeit in minute quantities of 3% and less were T-Man, 2- and 3-linked Man, and 3,4,6-linked Gal. GlcA was present only in the 4-linked form, in an amount (26%) which approximated that of 2,3-linked Man (22%) which as in the H. sericea study indicated the possibility of an inner core structure composed only of alternating GlcA and Man residues.

TABLE 34. METHYLATION ANALYSIS AND BASE DEGRADATION RESULTS FOR METHYLATED AHG-SD1-39

| PARTIALLY METHYLATED SUGAR | MOLAR PROPORTIONS[a] | | | |
|---|---|---|---|---|
| | I[b] | II[c] | III[d] | IV[e] |
| Mannitol[f] | - | - | - | 5 |
| [2]3,4,6-Man($d_2$)[g] | 3 | 2 | 5 | 4 |
| 2,3,4,6-Gal | 21 | 20 | 17 | 31 |
| [2],4,6-Man | 3 | 2 | 24 | 12 |
| 2,4,6-Gal | 24 | 23 | 22 | 19 |
| 4,6-Man | 21 | 22 | 4 | 2 |
| 2,3-Gal | - | - | tr | - |
| 3,4,6-Man | 2 | 3 | - | - |

Also present in minute proportions were 2,4-Gal, 2-Gal and 4-Gal

a Figures corrected for 26% GlcA

b I These results pertain to methylated AHG-SD1

c II These results pertain to methylated AHG-SD1-39

d III Dimsyl-treated methylated AHG-SD1-39

e IV DBU-treated methylated AHG-SD1-39

f Mannitol derivative has structure **10** (see Section 3.4.6)

g [ ] Indicates the position of the deuterium ($CD_3O$) i.e. O-2, this only applies to III and IV since I and II have the undeuterated 2,3,4,6- and 2,4,6-O-methyl mannoses.

Further evidence for the predominant presence in the core structure of the 2-O-( β -D-glucopyranosyluronic acid)-D-mannose aldobiouronic acid units was furnished by the treatment of methylated AHG-SD1-39 with potassium dimsyl and the non-nucleophilic base DBU (Table 34).

Qualitatively these studies showed that the GlcA was linked to Man through its O-2 position. Other important structural information obtained in this base degradation study using dimsyl and DBU included the following:

(i) There was a pronounced decrease in 2,3-linked Man (of 18% and 20% respectively) with a concomitant increase (of 22% and 10% respectively) in 2,4,6-Man (with the 2 position labelled with deuterium) and a small increase in T-Man (O-2 deuterated; 3% and 2%) with complete disappearance of the 3,4,6-Man; these results indicated that most of the GlcA was attached to the O-2 position of the 2,3-linked Man.

(ii) A mannitol, produced by the DBU method of GlcA degradation, had a structure identical to the product **10** shown to be present in the Smith degraded A-LS-SD1 of *H. sericea*. This indicated the presence of the sequence S-2M-4GlcA but no evidence was obtained for the presence of the GlcA-2M-4GlcA sequence. One reason for its absence could be the fact that complete degradation was not obtained during

these experiments. Optimisation of exposure time and other conditions will allow the full potential of this method to be utilised.

The major structural implication of the results obtained from this Smith degradation study and the characterisation of the various products is that the polysaccharide core structure could be composed either of alternating monomers or of oligomers of the aldobiouronic acid (GlcA-2Man) having either contiguous to them or interspersed between them one or more periodate-susceptible sugars, eg. a 2-linked Man or a 4-linked GlcA. The structure representing these features closely approximates that of structure **6**.
Further evidence for the existence of these long sequences of contiguous GlcA-2Man was sought by partial hydrolysis.

## 4.6 PREPARATION AND CHARACTERISATION OF GlcA-2Man FROM HG

This experiment was conducted under conditions similar to that used for the preparation of GlcA-2Man from HSLS (Section 3.7.5).
The isolated aldobiouronic acid (0.025 g), characterised by the usual methods, gave results essentially the same as those for GlcA-2Man in Table 11. N.m.r. characterisation utilising 1-D and 2-D methods showed anomeric proton signals similar to those obtained in the characterisation of this aldobiouronic acid from H. sericea gum (Section 3.5)[94].

## 4.7 COMPARATIVE PARTIAL HYDROLYSIS OF HG

The polysaccharide from HG was subjected to hydrolysis with sulphuric acid (Scheme 13) for 4 h, in a manner similar to that described for H. sericea (section 3.6). Ethanol precipitation yielded two products, one soluble in ethanol (yield 70% by wt.; neutral fraction), and the other insoluble (14% by wt.; the acidic fraction).

This study allowed preliminary comparison of the components of the polysaccharide from HG with those of H. sericea whole gum. Results obtained from the characterisation of the products of Smith-degraded HG (Table 30) indicated strong structural similarities to the H. sericea polysaccharide, viz. the presence of equal concentrations of GlcA and 2-linked Man concentrated in the innermost core structure, enveloped by many residues including, mainly, 3-linked Gal, 3,6-linked Gal and T-Gal (Section 4.3). Partial acid hydrolysis was, therefore, used as a means of verifying the existence of such a core structure. Comparison with H. sericea gum, utilising p.c. of the products of partial acid hydrolysis, gave the following information.

The acidic and neutral components of partially hydrolysed HG (Scheme 8) were separated by 2-dimensional p.c. using two different solvent systems (solvents B and C), which decreased the possibility of "loss" of some of the acidic components through avoidance of having to use extra solvent extraction

to remove the neutral components. Results were obtained showing that 6 of the 7 acidic components of partially hydrolysed HG had $R_{Gal}$ values (solvent C) almost identical to the 6 oligosaccharides of partially hydrolysed H. sericea gum. The extra oligosaccharide, $R_{Gal}$ 0.14, which does not have a linear relationship (according to the Bate-Smith and Westall method[95]), with the monomer and dimer is thus probably composed of residues different from those of the GlcA-2Man series.

The neutral fraction was shown by examination on 2-D p.c. to contain Gal (5+), Xyl (3+), Ara (2+), and 3 oligosaccharides with $R_{Gal}$ values (solvent B) 0.12 [0.52] (3+), 0.19 [0.63] (2+), and 0.23 [0.36] (2+), {figures in parentheses, [ ] are $R_{Gal}$ values in solvent C}; free mannose was absent. These monosaccharides were probably located on the periphery of the structure of the polysaccharide from HG, as was indicated by the autohydrolysis experiments.

The designations in Table 35 are strong possibilities for these components of HG. However,the question of their homogeneity must be answered before identities can be definite. Generally, the results obtained thus far from Smith degradation, autohydrolysis, and base degradation would almost corroborate these identities.

TABLE 35. COMPONENTS OF PARTIALLY HYDROLYSED PRODUCTS FROM HG AND H. sericea WHOLE GUM.

| $R_{Gal}$ values[a]: | | |
|---|---|---|
| HG | H. sericea | DESIGNATION[b] |
| 0.02 | 0.02 | Fraction 17 |
| 0.07 | 0.08 | Fraction 16 |
| 0.14 | - | ? |
| 0.23 | 0.22 | Dimer (**14**) |
| 0.43 | 0.42 | Mixture of Fractions 10 and 11 |
| 0.64 | 0.65 | Monomer (**3**) |
| 0.89 | 0.94 | GlcA |

a In solvent C
b All designations are relative to the products from H. sericea gum (Section 3.7.6)

Further experimentation, including characterisation after isolation of the fractions noted in this p.c. examination, would be necessary to furnish more information about the innermost structure of HG. Other methods would include column

chromatography, as in the H. sericea study, the more advanced HPLC method of isolation, and electrophoresis of these "spots" seen on p.c.

## 4.8 INVESTIGATION OF THE POLYSACCHARIDE EXUDATE FROM H. gibbosa. (ii) EXPERIMENTAL

### 4.8.1 Origin And Isolation

A freshly exuded sample of clear gum from Hakea gibbosa (HG) was collected on the slopes of the Constantiaberg mountains in Cape Town in March 1986. The gum (125 g) was dissolved with stirring in distilled water (10 L) at room temperature over a period of 48 h. After filtering through muslin the bark debris (1 g) was separated from the polysaccharide solution which was then concentrated and precipitated in ethanol. The polysaccharide, after dissolving in water and freeze-drying, yielded a white fluffy product (80 g; Table 28).

### 4.8.2 Methylation and Related Analyses

Methylation was performed according to the method described in Chapter 3.

A deionised sample of HG (0.1 g) was methylated by the method of Hakomori[67] followed by three Purdie treatments[66]. The product was purified as usual by chromatography on a column of Sephadex LH-20. Characterization of the methylated polysaccharide by g.l.c. and g.l.c.-m.s. of the hydrolysate, as the derived alditol acetates, gave the results shown in Table 29. Reduction with LAD allowed the linkages of the GlcA residues to be determined by this method.

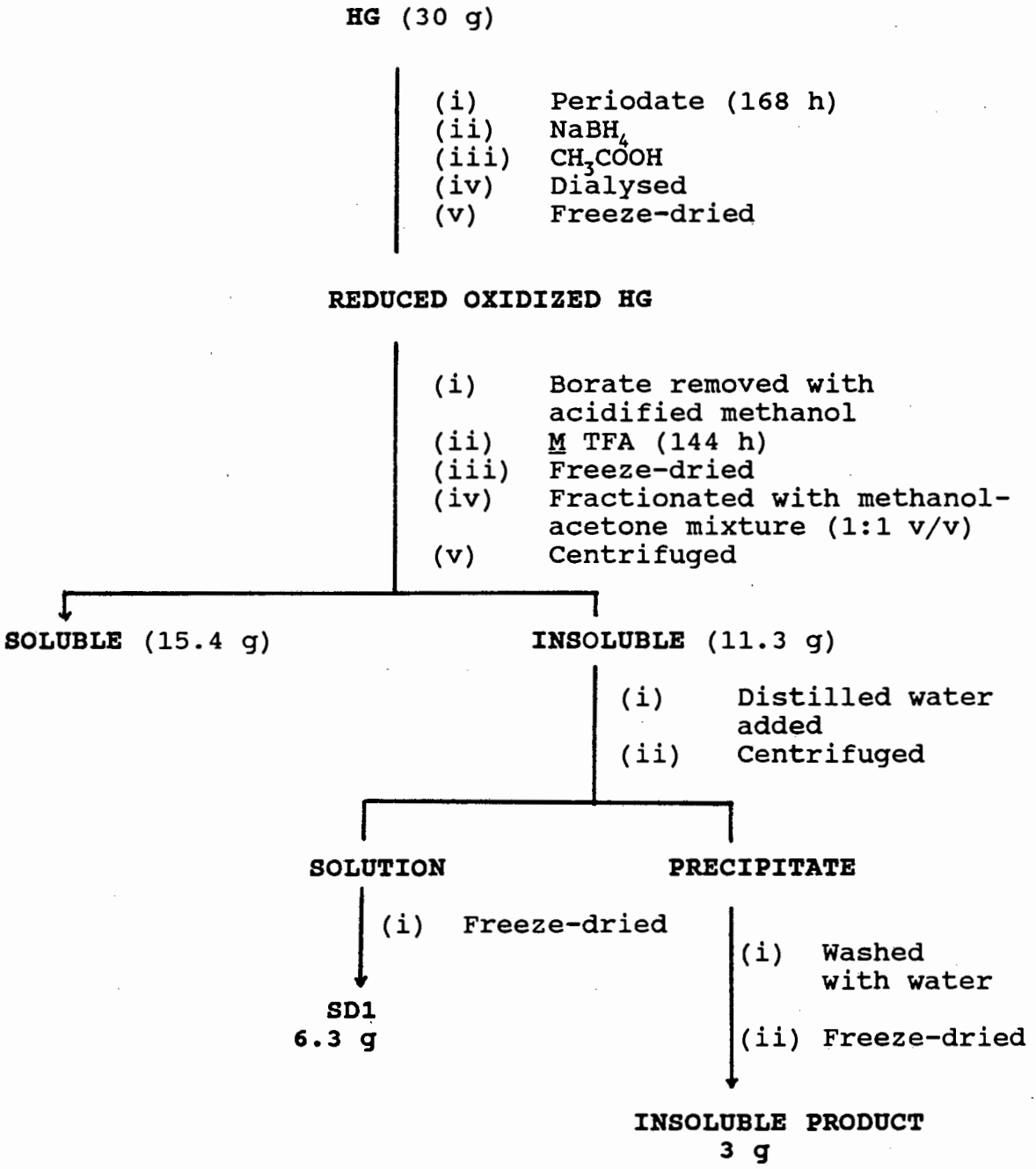
### 4.8.3 Smith Degradation of The Polysaccharide From *Hakea gibbosa* Gum

Smith degradation was performed according to the method previously discussed in Section 3.7.2.

#### 4.8.3.1 First Smith degradation (Scheme 9)

Oxidation of HG (30 g) for 168 h resulted in a periodate consumption of 6.2 mmol $g^{-1}$ which was *ca*. 5% greater than the calculated value (5.9 mmol $g^{-1}$). The Smith degradation, performed according to Scheme 9, yielded the reduced-oxidized polysaccharide and eventually two products on methanol-acetone fractionation. The fraction soluble in 1:1 (v/v) methanol-acetone was examined by p.c. (solvents A, B and C) using both p-anisidine hydrochloride and alkaline silver nitrate as detection reagents.

SCHEME 9 FIRST SMITH DEGRADATION OF HG



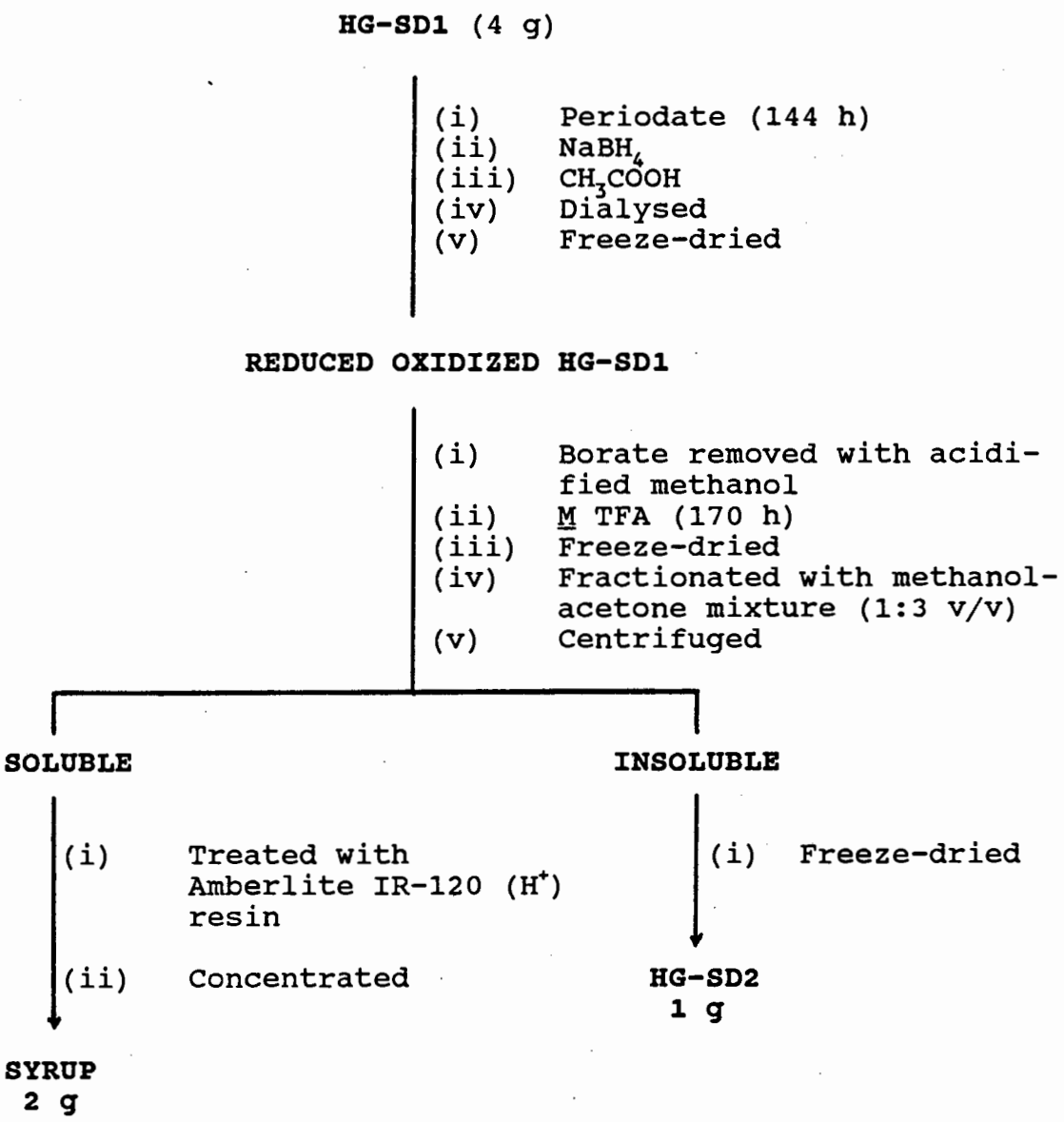
The fraction insoluble in methanol-acetone was suspended in water giving two fractions, one water-insoluble (3.0 g) and the other soluble (HG-SD1; 6.3 g); both were characterised and found to have identical sugar compositions. Further experimentation was performed on the water-soluble fraction HG-SD1).

#### 4.8.3.2 **Second Smith degradation of HG (Scheme 10)**

A sample of HG-SD1 (4 g; Table 30) was subjected to periodate oxidation with the usual arsenite monitoring. After 144 h the periodate consumption became constant at 6.1 mmol $g^{-1}$, which was 0.02 mmol $g^{-1}$ higher than the theoretically calculated value. This over-oxidation was not considered to be significant. After the procedure shown in Scheme 10 had been followed, the HG-SD2 product was characterised (Table 30). Exposure of HG-SD2 to M TFA for 40 days removed all of the glycolaldehyde (1%). After this exposure no change in $\bar{M}_w$ was noted on examination by s.e.c. (Sepharose 4B).

The fraction that was soluble in the 1:3 methanol-acetone mixture used to isolate HG-SD2 was deionised in the usual manner and evaporated to a syrup, was which examined by p.c. (solvents A, B and C), using both p-anisidine HCl and alkaline $AgNO_3$ as detection reagents.

SCHEME 10 SECOND SMITH DEGRADATION OF HG



### 4.8.4 **Partial Hydrolysis (Autohydrolysis) of HG Yielding AHG (Scheme 11)**

A vacuum-dried sample (45 g) of the polysaccharide from H. gibbosa gum was subjected to partial hydrolysis with 0.03 M $H_2SO_4$ in a manner similar to that described for HSWS (section 3.7.3) with monitoring by polarimetry (Fig. 25). Ethanol precipitation gave two products, viz. an insoluble (AHG; 24 g) and a soluble fraction (11 g). The insoluble fraction was thoroughly washed with ethanol, centrifuged and the supernatant added to the soluble fraction which was then concentrated to a syrup. The syrup on examination by p.c., using solvents A, B and C, was shown to contain the components: Ara (4+), Xyl (tr), Gal (tr) and in solvent D also slower-moving components with $R_{Gal}$ values 0.08 (+), 0.18 (+) and 0.36 (+).

The ethanol-insoluble fraction was dissolved in water and freeze-dried to yield a pink fibrous product (AHG) which was characterised (Table 31).

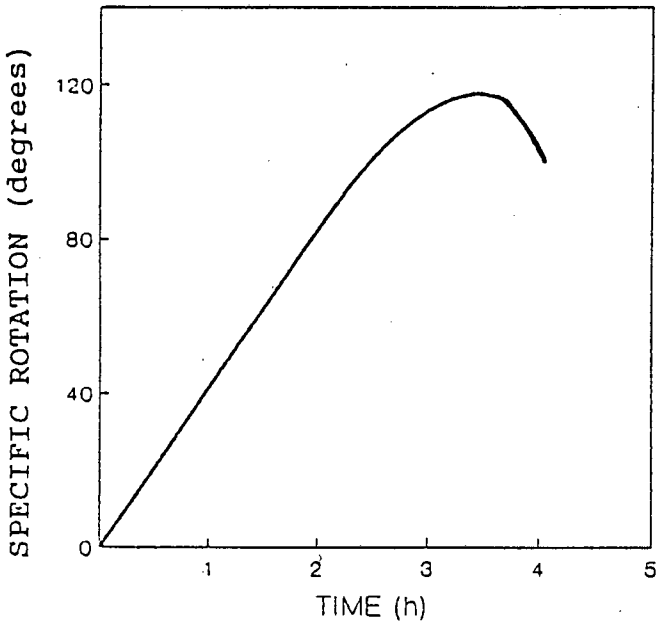
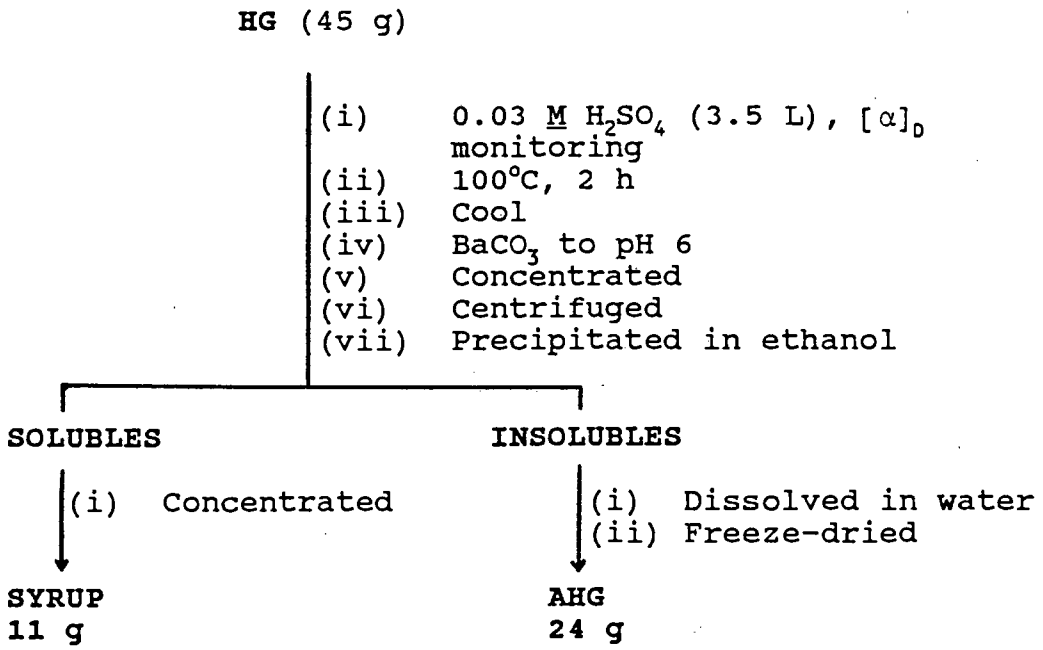



FIGURE 25. MONITORING BY $[\alpha]_D$ OF PARTIAL HYDROLYSIS

SCHEME 11 AUTOHYDROLYSIS OF HG



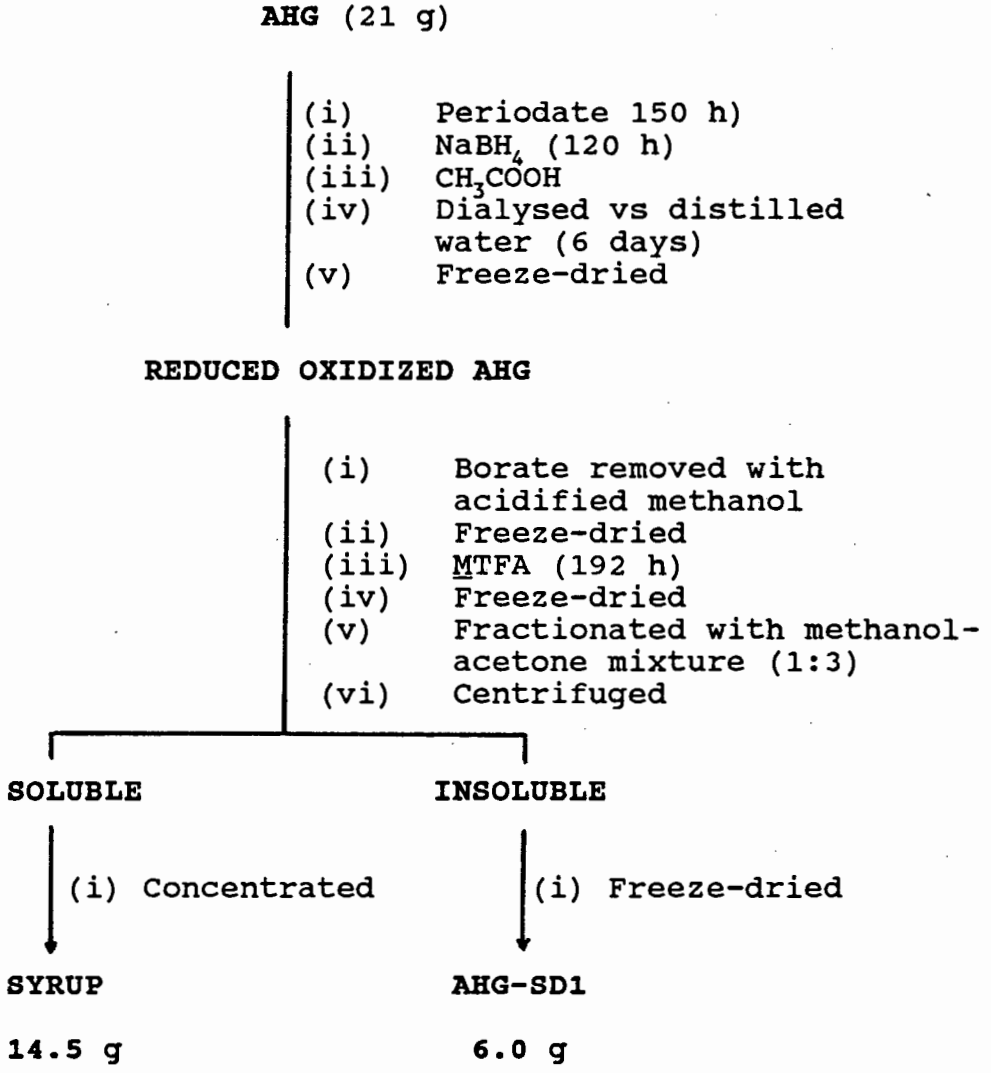
#### 4.8.4.1 **Smith degradation of AHG (Scheme 12)**

A sample of AHG (21 g) was oxidized for 150 h, after which time the consumption of periodate became constant at a value of 8.6 mmol $g^{-1}$, not significantly different from the theoretical value of 8.75 mmol $g^{-1}$ calculated from methylation data (Table 32). The Smith-degradation procedure used is shown in Scheme 12.

The methanol-acetone soluble fraction was deionised in the usual way and examined by p.c. (solvents A, B and C). The AHG-SD1 was characterised (Table 33) and shown to contain glycolaldehyde 1%. Treatment of AHG-SD1 with M TFA (39 days) yielded the product AHG-SD1-39, devoid of glycolaldehyde which showed a decreased $\bar{M}_w$ of 5000. LAD reduction of MHG-SD1 showed the GlcA to be present in the 4-linked form.

Base degradations of methylated AHG-SD1-39 were performed with potassium dimsyl and DBU as described in Section 2.8 (results in Table 34).

SCHEME 12 SMITH DEGRADATION OF AHG



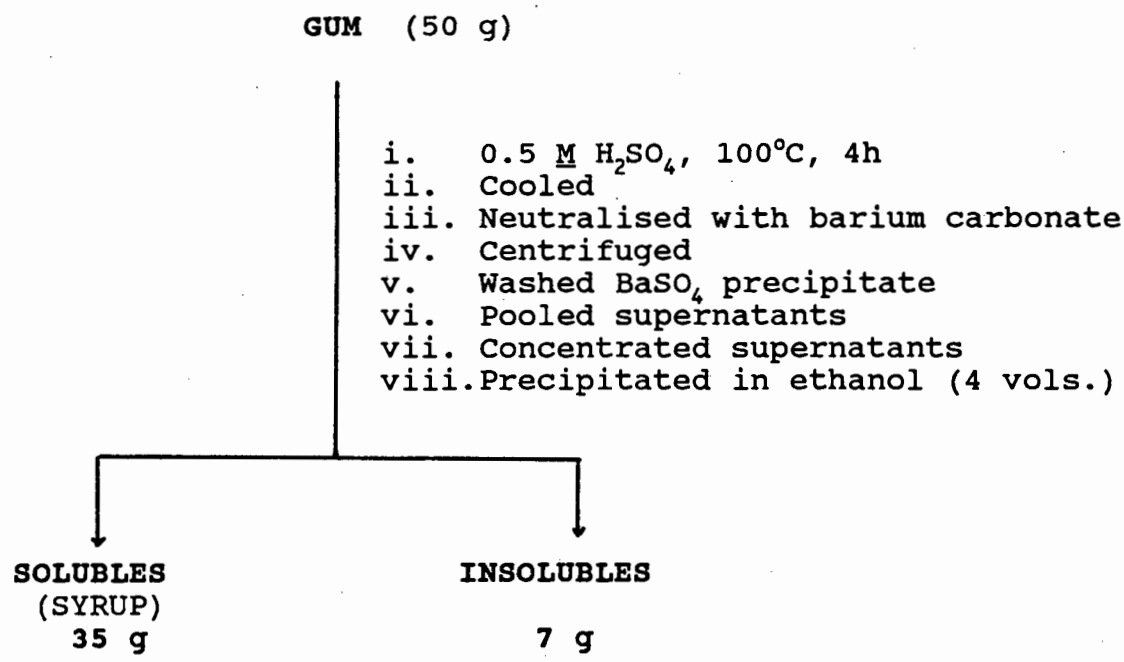
### 4.8.5. **Preparation and Characterisation of GlcA-2Man (3) from HG**

The aldobiouronic acid was prepared and isolated in a manner only slightly different to that previously discussed for HSWS in Section 3.5. In this study two fractions were isolated after hydrolysis and neutralisation, viz. one insoluble in ethanol (0.4 g) and the other soluble in ethanol (3.0 g). Only the latter syrup was further investigated. It was subjected to preparative chromatography on a column of Sephadex G-10 using water as an eluent. The eluted components were the aldobiouronic acid (yield 0.5%), oligosaccharides with $R_{Gal}$ (solvent C) values of 0.01 (+), 0.08 (tr), 0.14 (+), and 0.43 (tr) and Ara(++), Xyl (+), and Gal (++).
The isolated aldobiouronic acid (0.025 g) was characterised by the usual methods and shown to be GlcA-2Man.

### 4.8.6. **Comparative Partial Hydrolysis Of Hakea gibbosa Gum (Scheme 13)**

Partial hydrolysis was conducted according to the method described in section 3.7.6., in which the polysaccharide (50 g) was refluxed at 100°C with sulphuric acid (0.5 M, 4 h, 700 mL), neutralised and cooled. Concentration followed by precipitation in ethanol yielded 2 products, one soluble in ethanol (35 g) and the other insoluble (7 g).

SCHEME 13 PARTIAL HYDROLYSIS OF HG




The soluble product was concentrated to a syrup and examination by p.c. revealed the presence of Ara (4+), Xyl (tr), Gal (4+), and 2 oligosaccharides with $R_{Gal}$ values (solvent C) of 0.51 and 0.63, corresponding to the disaccharides Gal-6Gal and GlcA-2Man. The insoluble product was subjected to 2-dimensional p.c. on Whatman filter paper (50 x 50 cm) utilizing solvent C in the first run, then solvent B in the second run, orthogonal to the first. The product was shown to be composed of at least 7 acidic components with $R_{Gal}$ values

(solvent C) shown in Table 35, and 5 components which in solvent B had $R_{Gal}$ values of 0.12 (+++), 0.19 (++) and 0.23 (++), Gal (+) and Xyl (tr).

Hydrolysis of the insoluble fraction with 2 M TFA at 100°C for 18 h, followed by examination on p.c. (solvent C), revealed the presence of Gal (++), and Man (++); GlcA content (+++) was determined colorimetrically[85].

## 5. THE *Hakea* GUMS: CONCLUSION

### 5.1 Introduction

The polysaccharide gums from the Hakea species *H. sericea* and *H. gibbosa* have been shown to have close structural similarities. These large polysaccharide molecules contain a core structure of GlcA and Man surrounded by an arabino-xylogalactan envelope. The isolation of a dimer (**14**) and a substituted trimer (**16**) of the aldobiouronic acid unit among the products of partial acid hydrolysis of *H. sericea* gum has shown the presence of sequences of 2 or 3 contiguous units in the core, with galactosyl branches linked to O-3 of Man and sometimes to O-3 of GlcA. There is evidence to suggest that xylose is linked to some of the 4-linked GlcA through O-3 in *H. sericea* gum unlike leiocarpan gum[20] where it is joined to the O-6 position of Man.

A summary of the structural features found (mentioned in detail in section 4) and also the methods used to substantiate them, is presented below.

### 5.2. General summary of substituents

The polysaccharide gums of the Hakeas are composed mainly of galactose, with smaller proportions of Ara, and Man and Xyl (molar ratio 1:1) as minor components. GlcA and Man were

found in equal proportion in H. sericea gum and in molar ratio 2:1 in that of H. gibbosa. Methylation analyses of the native polysaccharide gums revealed the major galactosyl residues to be 3,6-and 3-linked Gal, in molar ratio 2:1 in H. sericea gum and 1:1 in that of H. gibbosa. Both polysaccharide gums have Gal, Xyl and Araf end groups attached to arabinogalactan side chains composed mostly of 3,6-linked Gal residues. Autohydrolysis allowed peeling of the Gal, Xyl and Araf residues from the periphery of the large structure within which a more resistant inner structure is enveloped. Stronger acid was used successfully to cleave the innermost core of the polysaccharide. Characterization of the resultant oligosaccharides confirmed the presence of Gal-3Gal and Gal-6Gal in the neutral fraction of the dialysate, and analysis of the isolated acidic oligosaccharides[3,14-16] allowed the structural implications of the Smith-degradation experiments to be verified, viz. that there are sequences of 4-linked GlcA and 2-linked Man in the core. The presence of an abundance of the GlcA-2Man aldobiouronic acid was noted on acid hydrolysis with TFA and $H_2SO_4$ which also illustrated the resistance to acid of the glucuronomannosyl bond. The dimer in H. gibbosa gum was identified by comparative p.c. of the partial acid hydrolysate, using the dimer of H. sericea as a standard. In the gum from H. sericea the trimer seems to be present.

Substitution of this glucuronomannanglycan type core with mainly Gal residues has been shown, by methylation analysis before and after LAD reduction, to occur mainly at O-3 of Man and sometimes at O-3 of GlcA.

G.lc.-m.s. following base degradation of various products of the gums revealed the presence of specifically labelled mannitols (either acetylated or deuteriomethylated at points of linkage) indicating the sequences : GlcA-2Man-Sugar and Sugar-2Man-4GlcA. The expected evidence for GlcA linked interiorly as well as exteriorly to Man was not obtained from degradation with the non-nucleophilic base. However proof for the existence of this sequence in both Hakea polysaccharide gums, albeit in low concentration, was obtained from partial hydrolyses.

The results from Smith degradations and autohydrolyses indicated firstly that Man and GlcA residues become concentrated in products isolated from the innermost core, and secondly that they occur in a molar ratio of 1:1 in this core. Thus, the sugars involved in these innermost parts of the polysaccharide structure were shown to be Gal, Man and GlcA with the latter 2 components in equal amounts, and the Gal in lesser amounts.

Thus, cores of both Hakea gums were shown to be very similar, although some differences were noted :

(i) The presence of some Xyl in the innermost core of H. sericea, probably linked through O-3 to GlcA;

(ii) The proportion of GlcA appearing to be double that of Man in the native polysaccharide of H. gibbosa, whereas these residues were equimolar in H. sericea gum ;

(iii) The occurrence of mostly 3,4-linked GlcA residues in the native polysaccharide gum of H. gibbosa, whereas 4-linked GlcA was present in equimolar proportion in H. sericea gum; these were also shown to be equal in the autohydrolysis products of both gums);

(iv) H. gibbosa gum contained 50 % more Araf present as end group.

Structure **6** is a good example of an average molecule for H. sericea gum with the polysaccharide gum from H. gibbosa differing in the respects mentioned above.

In Fig. 26 a proposed "average" structure for the Hakea gums is presented, and some of the methods that allowed characterization of specific features are indicated.

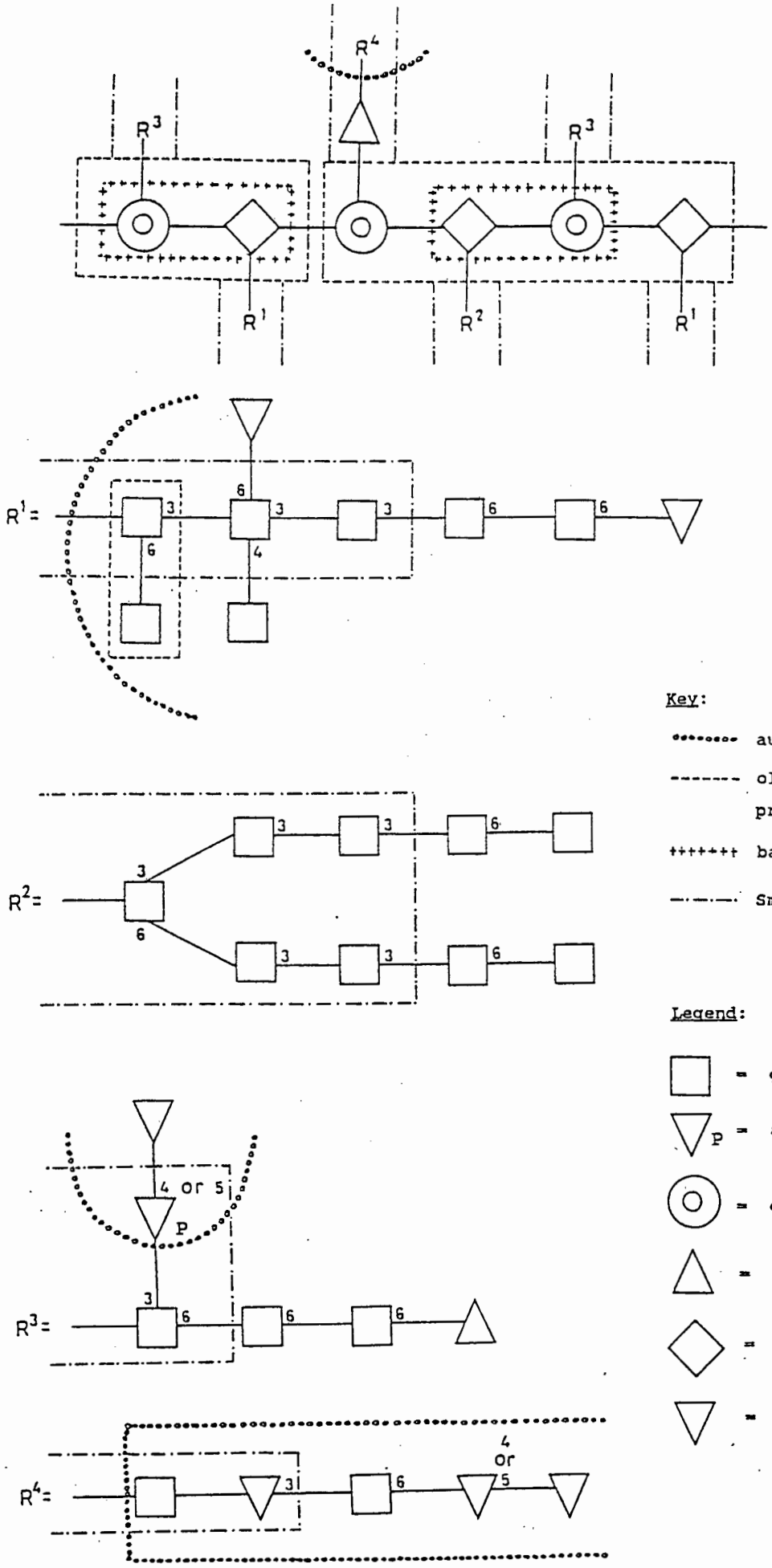



FIGURE 26. AN AVERAGE STRUCTURE FOR THE TWO POLYSACCHARIDE GUMS FROM *Hakea* SPECIES

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